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CLOSTRIDIUM DIFFICILE INFECTIONS AND THEIR TREATMENT

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ACADEMIC DISSERTATION

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CONTENTS

Abstract.....	6
List of Original Publications.....	8
Abbreviations	9
1 Introduction.....	10
2 Review of the literature.....	12
2.1 Discovery of <i>C. difficile</i> and its role in antibiotic-associated diarrhea	12
2.2 Definition of CDI.....	13
2.3 Epidemiology of CDI	14
2.3.1 Changing epidemiology.....	14
2.3.2 Community associated CDI	15
2.3.3 Burden of CDI	16
2.3.4 CDI in Special Populations	17
2.4 Pathogenic features of <i>C. difficile</i>	19
2.4.1 Pathogenesis	19
2.4.2 Virulence factors	19
2.5 Risk factors of CDI.....	22
2.5.1 Risk factors for disease.....	22
2.5.2 Risk factors for recurrent CDI	24
2.5.3 Risk factors for adverse outcome	25
2.6 Clinical features of CDI	25
2.6.1 Asymptomatic carriage	26
2.6.2 Mild to moderate CDI	26
2.6.3 Severe CDI.....	26
2.6.4 Fulminant CDI	27
2.6.5 Recurrent CDI.....	27
2.6.6 Differential diagnosis of CDI	28
2.6.7 Extraintestinal CDI.....	29
2.7 Diagnosis of CDI	29
2.7.1 Laboratory diagnosis.....	29
2.7.2 Role of endoscopy and radiology.....	30

2.8	Treatment of CDI.....	31
2.8.1	Treatment of a first episode of CDI.....	33
2.8.2	Treatment of severe, complicated CDI	34
2.8.3	Surgery for complicated CDI	35
2.8.4	Treatment of recurrent CDI.....	36
2.8.5	Treatment of third and subsequent relapses.....	36
2.8.6	Fecal microbiota transplantation	37
2.8.7	Role of probiotics.....	38
2.8.8	Immunotherapy.....	38
2.9	Infection prevention and control.....	39
3	Aims of the study	41
4	Patients and methods.	42
4.1	Patients	42
4.2	Study designs	43
4.3	Definitions	43
4.4	Study treatments.....	44
4.5	Microbiological methods.....	45
4.6	Ethical aspects.....	46
5	Results.....	47
5.1	Patient characteristics (I-III)	47
5.2	Response to therapy	48
5.3	Adverse events	50
5.4	Extra-intestinal CDI	50
6	Discussion	52
6.1	CDIW and metronidazole for recurrent CDI	52
6.2	Fecal microbiota transplantation for recurrent CDI	53
6.3	Rifaximin for recurrent CDI	54
6.4	Extra-intestinal CDI	56
6.5	Remaining challenges	57
7	Summary and Conclusions	59
8	Acknowledgements.....	60
9	References	62
	Original Publications.....	97

To my family

ABSTRACT

Background and aims. Standard treatment of recurrent *Clostridium difficile* infection (CDI) with antibiotics leads to recurrences in up to 50% of patients. In recent years the incidence and mortality of *Clostridium difficile* (*C. difficile*) enteritis have increased. Nevertheless, *C. difficile* has rarely been isolated in extra-intestinal infections. The aims of the study were to investigate efficacy of rifaximin, metronidazole, fecal microbiota transplantation (FMT) and *Clostridium difficile* immune whey (CDIW) in the treatment of recurrent CDI and to characterize clinical feature and risk factors for extra-intestinal CDI.

Subjects and methods. Study I was a prospective, randomized, double-blind study designed to compare CDIW with metronidazole for treatment of laboratory-confirmed, mild to moderate episodes of recurrent CDI. CDIW was manufactured by immunization of cows in their gestation period with inactivated *C. difficile* vaccine. The resulting colostrum was processed, immunoglobulins were concentrated and the end-product containing high titres of *C. difficile* immunoglobulin was used as CDIW. 20 patients received metronidazole at a dosage of 400 mg t.i.d. and 18 patients CDIW 200 ml t.i.d. Study II was a retrospective review of 70 patients with recurrent CDI who had undergone fecal transplantation. FMT was performed at colonoscopy by infusing fresh donor feces into cecum. Before transplantation, the patients had whole-bowel lavage with polyethylene glycol solution. Study III was a retrospective study of 32 patients who were treated with rifaximin for recurrent CDI. In Study IV extra-intestinal CDIs were searched for in an electronic database of all *C. difficile* positive isolates found during a 10-year period. The medical records were reviewed retrospectively. Disease severity and co-morbidities of the patients were evaluated using Horn disease severity and Charlson co-morbidity indexes.

Results. In Study I, 10 weeks after the beginning of treatment, sustained responses were observed in 11 (55%) of 20 patients receiving metronidazole and 10 (56%) of 18 patients treated with CDIW. In Study II, 12 weeks after FMT, 66 (94%) of 70 patients had a favourable response. In Study III, 12 weeks after rifaximin treatment 17 (53%) of 30 patients had no relapse. In Study IV extra-intestinal CDI was found in 31 patients who comprised 0.17% of all CDIs. One-year mortality rate was 36% and it correlated with the severity of underlying diseases.

Conclusions. CDIW was as effective as metronidazole in the prevention of CDI recurrences and it was well tolerated. FMT through colonoscopy seems to be an effective treatment also for recurrent CDI caused by the virulent *C. difficile* 027 strain. The MIC value of rifampin seemed to predict the response to rifaximin treatment. Extra-intestinal CDIs occur mainly in hospitalized patients with significant comorbidities. Extra-intestinal CDIs in the abdominal area may result either from intestinal perforation after infection or after intestinal surgery. *C. difficile* may reach distant sites via bacteremia. Mortality in extra-intestinal CDIs is associated with the severity of underlying diseases.

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, referred to in the text by their Roman numerals:

- I Mattila E, Anttila VJ, Broas M, Marttila H, Poukka P, Kuusisto K, Pusa L, Sammalkorpi K, Dabek J, Koivurova OP, Vähätalo M, Moilanen V, Widenius T. A randomized, double-blind study comparing *Clostridium difficile* immune whey and metronidazole for recurrent *Clostridium difficile*-associated diarrhoea: Efficacy and safety data of a prematurely interrupted trial. *Scand J Infect Dis.* 2008;40(9):702-8.
- II Mattila E, Uusitalo-Seppälä R, Wuorela M, Lehtola L, Nurmi H, Ristikankare M, Moilanen V, Salminen K, Seppälä M, Mattila PS, Anttila V-J, Arkkila P. Fecal Transplantation, Through Colonoscopy, Is Effective Therapy for Recurrent *Clostridium difficile* Infection. *Gastroenterology* 2012;142:490-6.
- III Mattila E, Arkkila P, Mattila PS, Tarkka E, Tissari P, Anttila VJ. Rifaximin in the treatment of recurrent *Clostridium difficile* infection. *Aliment Pharmacol Ther.* 2013;37(1):122-8.
- IV Mattila E, Arkkila P, Mattila PS, Tarkka E, Tissari P, Anttila VJ. Extra-intestinal *Clostridium difficile* infections. *Clin Infect Dis.* 2013;57(6):e148-53.

ABBREVIATIONS

AAD	antibiotic-associated diarrhea
AAHC	antibiotic-associated haemorrhagic colitis
ACG	American College of Gastroenterology
ADM	agar dilution method
AIM	agar incorporation method
ANTI-TNF	anti-tumor necrosis factor
CA	community associated
CCFA	cycloserine-cefoxitin-fructose
CCNA	Clostridium difficile cytotoxin neutralization assay
CDI	Clostridium difficile infection
CDIW	Clostridium difficile immune whey
CDT	Clostridium difficile transferase
CT	computed tomography
EIA	enzyme immunoassay
EMA	European Medicines Agency
ESCMID	European Society of Clinical microbiology and Infectious Diseases
FAA	Fastidious Anaerobe Agar
FDA	Food and Drug Administration (USA)
FMT	fecal microbiota transplantation
HUCH	Helsinki University Central Hospital
HUSLAB	Helsinki University Central Hospital laboratory diagnostics
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IDSA	Infectious Diseases Society of America
IVIG	intravenous immunoglobulin
LOS	length of stay
Paloc	pathogenicity locus
PCR	polymerase chain reaction
MIC	minimum inhibitory concentration
RCDI	recurrent Clostridium difficile infection
SAE	serious adverse event
SHEA	Society for Healthcare Epidemiology of America
TC	toxigenic culture
t.i.d.	three times in a day
VRE	vancomycin resistant enterococcus
WBC	white blood cell

1 INTRODUCTION

Clostridium difficile infection (CDI) is a common cause of both community- and hospital-acquired diarrhea, usually occurring after exposure to antibiotics. During the past few years, *C. difficile* infection has become more frequent, more severe, more refractory to standard treatment, and more likely to relapse (Gravel et al. 2009, Zillberg et al. 2010, Pepin et al. 2005¹, Musher et al. 2005). Recurrent CDI increases the average length of hospitalization and cost of treatment. In addition, patients often become frustrated by the consistent reappearance of symptoms and the repeated need for treatment. Also recurrent CDI is associated with severe complications of megacolon, perforation, shock, or death (Pepin et al. 2006).

Current treatment with metronidazole or vancomycin against CDI is suboptimal, especially in terms of high recurrence rates. Both of these antibiotics alter the normal gut flora that provides colonization resistance against *C. difficile* (Chang et al. 2008). A number of empirical approaches have been used to treat recurrent CDI. New antibiotics have been introduced including rifaximin (Johnson et al. 2007), nitazoxanide (Musher et al. 2006) and fidaxomicin (Louie et al. 2009). Passive immune therapy has been used as intravenous immunoglobulin (Wilcox 2004). Also, probiotic regimens with *Saccharomyces boulardii* (Surawicz et al. 2000) and *Lactobacillus* (Wullt et al. 2003) have been used. All these currently available treatment modalities have limited efficacy. Data are lacking to support any particular treatment strategy.

Treatment of CDI enteritis is shifting towards local therapy such as per oral non-absorbable antibiotics vancomycin, rifaximin, and fidaxomicin. (Lo Vecchi and Zacur 2012). Although *C. difficile* enteritis is the most frequent presentation of CDI, *C. difficile* causes infections also outside the intestine. In clinical practice, a finding of *C. difficile* in an extra-intestinal site is often a surprise. Evaluation of the significance of the finding may not always be straightforward especially when *C. difficile* is found together with other microbes.

After the appearance of ribotype 027 in Finland (Lyytikäinen et al 2007) there were more patients with relapses of CDI and the relapses were also more difficult to treat with conventional antibiotic therapy for CDI. This encouraged the use of *Clostridium difficile* immune whey (CDIW), rifaximin and fecal transplantation for recurrent CDI, and they became a treatment option for selected patients.

The present studies were undertaken to gain insight into the effect and safety of these treatment modalities. We conducted a nationwide, double-blind, multicentre study comparing CDIW and metronidazole in recurrent CDI (RCDI). We also conducted a retrospective study of 70 patients with RCDI treated with colonoscopy

administered fecal microbiota transplantation (FMT) in 5 different centers. The FMTs were performed using a standard method in all centers. We retrospectively evaluated the records of 32 patients who were treated with rifaximin for RCDI. We also performed a systematic analysis of all consecutive extra-intestinal CDIs during 10 years time in order to characterize predisposing factors, clinical features and outcomes of these infections. In addition, clinical features and risk factors of extra-intestinal CDI were characterized.

2 REVIEW OF THE LITERATURE

2.1 DISCOVERY OF *C. DIFFICILE* AND ITS ROLE IN ANTIBIOTIC-ASSOCIATED DIARRHEA

In 1935, Hall and O'Toole first isolated a gram-positive, cytotoxin producing anaerobic bacterium from the normal intestinal flora of newborn infants (Hall and O'Toole 1935). They named it *Bacillus difficilis* to reflect the difficulties they encountered in its isolation and culture. These investigators also showed that this organism produced a toxin that was highly lethal to mice. Almost 40 years later the species now known as *C. difficile* was identified as the etiological agent of antibiotic-associated pseudomembranous colitis (Bartlett et al. 1978). Paradoxically, the major pathologic feature of antibiotic associated colitis, pseudomembranous colitis, was first described in 1893 (Finney 1893) in the preantibiotic era.

Antibiotic-associated diarrhea (AAD) became a well-recognized complication of antibiotic use shortly after the introduction of these agents in the early 1950s. The incidence of AAD varies from 5% to 39% depending upon the population (McFarland 1998) and type of antibiotic (Bartlett 2002). The rates of diarrhea associated with parenterally administered antibiotics, especially those with enterohepatic circulation, are similar to rates associated with orally administered agents (Wiström et al 2001).

In patients who develop AAD due to *C. difficile*, administration of antibiotics either allows colonization by *C. difficile* after ingestion of environmental spores or permits overgrowth of indigenous *C. difficile* (Wilson 1993). Approximately 20-30% of cases of AAD, 50-75 % of those with antibiotic-associated colitis, and in more than 90 % of those with antibiotic associated pseudomembranous colitis are due to *C. difficile* infection (CDI) (McFarland 1998, Bartlett 2002).

The etiology of AAD and colitis that is *not* caused by *C. difficile* is poorly understood. *Candida spp*, particularly among elderly hospitalized patients, enterotoxigenic *Clostridium perfringens* and *Klebsiella oxytoca* has been cited as possible cause of AAD (Levine et al. 1995, Asha and Wilcox 2002). Recent antibiotic exposure has emerged as a distinct factor of in both sporadic cases and outbreaks of salmonellosis (Neal et al 1994). These organisms are rare causes, however, and 70%–80% of AAD cases have no established microbial pathogen. Many cases are probably episodes of osmotic diarrhea resulting from the failure of the fecal flora to catabolize carbohydrates (Young VB and Schmidt 2004) or changes in short-chain fatty acid metabolism (Hove et al 1996).

2.2 DEFINITION OF CDI

The Society for Healthcare Epidemiology of America/Infectious Diseases Society of America (SHEA/IDSA) guidelines (Cohen 2012 et al) define CDI in the following manner: A case definition of CDI should include the presence of symptoms (usually diarrhea) and either a stool test result positive for *C. difficile* toxins or toxigenic *C. difficile*, or colonoscopic findings demonstrating pseudomembranous colitis. In the ESCMID (European Society of Clinical Microbiology and Infectious Diseases) guideline (Bauer et al. 2009) CDI is defined as a clinical picture compatible with CDI and microbiological evidence of toxin-producing *C. difficile* in stool without evidence of another cause of diarrhoea or pseudomembranous colitis. CDI may be further defined according to the time of symptom onset and history of hospitalization (Table 1).

Table 1 Definition of CDI

Type of case	Definition
Health-care facility-onset health-care facility associated (HO-HCFA)	Occurs when onset of symptoms 3 days after admission to a health-care facility
Community onset healthcare facility associated (CO-HCFA)	Onset of symptoms within 4 weeks after being discharged from a health-care facility.
Community associated (CA)	Occurs when onset of symptoms occurs outside a health-care facility or < 3 days after admission to a health-care facility and has not been discharged from a healthcare facility in the previous 12 weeks
Indeterminate or unknown onset (ID)	CDI develops after being discharged from a health-care facility 4 – 12 weeks previously
Recurrent CDI	Episode of CDI that occurs 8 weeks after the onset of a previous episode, provided the symptoms from the previous episode resolved

2.3 EPIDEMIOLOGY OF CDI

2.3.1 CHANGING EPIDEMIOLOGY

Since the early 2000s, the epidemiology of CDI has changed dramatically across the Europe, United States and Canada; an increase in overall incidence has been highlighted by outbreaks of more-severe disease than previously observed (McDonald et al. 2006, Redelings et al 2007, Burckhardt et al. 2008, Gravel et al. 2009). CDI outbreaks often correlate with increasing total antimicrobial consumption, introduction of a particular strain of *C. difficile*, poor attention to environmental cleaning and waning compliance with good infection control practices (Owens et al. 2008).

The rising rates of CDI have been attributed to the presence of ribotype 027 strain but are not limited to the spread of this strain. Most of the evidence suggesting that 027 strains are more virulent and associated with more severe disease are derived from studies conducted during outbreak settings. In contrast, 027 strains were not found to be more virulent in studies conducted in non-epidemic settings or in settings where the prevalence of 027 remains low (Sirard et al. 2011). Historic and recent isolates of the 027 strain differ in their level of resistance to fluoroquinolones; more recent isolates are more highly resistant to these drugs (McDonald et al. 2005). This, coupled with increasing use of the fluoroquinolones likely promoted dissemination of a once uncommon strain. In some countries in Europe the prevalence of the 027 strain is now decreasing (Hensens et al. 2009, Bauer et al. 2011). Depending on the country, other emerging PCR-ribotypes have also been reported and include 012, 017, 019, 036, 078 and 153 (Knetsch et al. 2011, Dawson et al. 2009). Ribotype 078 causes disease both in animals, particularly calves and pigs, and humans. Studies to date have shown a high degree of genetic relatedness in the animal and human strains (Goorhuis et al. 2008). In the Netherlands, patients infected with ribotype 078 were younger (67.4 versus 73.5 years) and had community associated disease more frequently (17.5% versus 6.7%) than patients infected with ribotype 027 (Debast et al. 2008).

It appears that increases in the rate of CDI hospital discharges in USA may be leveling off, with a 2.5% decrease in the point estimate from 8.75 per 1000 discharges in 2008 to 8.53 per 1000 discharges in 2009 (Lucado et al 2012). The same phenomenon is also observed in Finland (Kanerva et al 2013). The first case of fatal *C. difficile* ribotype 027-associated disease was detected in Finland in October 2007 (Lyytikäinen et al 2007). Since then, the National Public Health Institute intensified the surveillance and control of CDI. In January 2008, laboratory-based surveillance of *C. difficile* was initiated as a part of the Finnish National Infectious Disease Register (NIDR) and enhanced surveillance of hospitalized patients with CDI by the Finnish Hospital Infection Programme (SIRO). Both

the population-based incidence of *C. difficile* and the enhanced surveillance of hospitalized patients with CDI showed a decrease by one-quarter during the first years of surveillance in 2008-2010 (Kanerva et al. 2013). Since then, the annual incidence is stabilized, as seen in Figure 1. In Finland 5257 toxin positive patients were reported for National Institute for Health and Welfare in 2012.

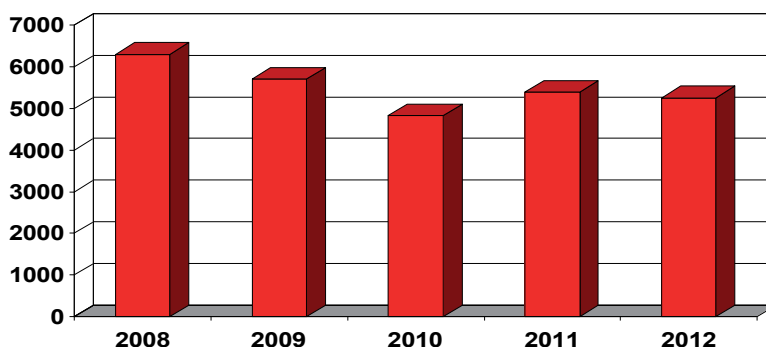


Figure 1 Annual incidence of CDI in Finland. Source: National Institute for Health and Welfare.

2.3.2 COMMUNITY ASSOCIATED CDI

The incidence of CDI might be increasing among persons living in the community, including, but not limited to, healthy persons without recent healthcare contact (Kyne et al. 1998, Johal et al. 2004, Dial et al. 2005). Data from the United States, Canada, and Europe suggest that approximately 20%–27% of all CDI cases are community associated, with an incidence of 20–30 per 100 000 population (Wilcox et al. 2008, Kutty et al. 2010, Lambert et al. 2009). Compared with hospital acquired infections, patients with community associated CDI are younger, healthier and less likely to have been exposed to antibiotics (Khanna 2012²). These cases stress the importance of considering CDI in the differential diagnosis of any patient with diarrhea, even in the absence of traditional risk factors. The high incidence of CDI in health care facilities compared with the community presumably results from the high density of individuals prone to CDI, classically, elderly patients with comorbidity, who may serve as a reservoir in which *C. difficile* can amplify.

Possible community sources for CDI include soil, water, pets, meats and vegetables (Hensgens et al. 2012²). Direct transmission of *C. difficile* from animals, food or the environment to humans has not been proven, although similar

ribotypes are found. As no outbreaks of CDI have been reported among humans in the community, host factors that increase vulnerability to CDI might be of more importance than increased exposure to *C. difficile*.

2.3.3 BURDEN OF CDI

CDI is a leading cause of hospital associated infectious diarrhea (McFarland et al 1989). Recent data from 28 community hospitals in the southern United States suggest that *C. difficile* has replaced methicillin-resistant *Staphylococcus aureus* as the most common cause of healthcare-associated infection (Miller et al 2011¹). The annual cost of hospital care for patients with CDI totals to approximately \$3.2 billion in the USA (O'Brien et al. 2007), although a significant percentage of CDI cases are missed because clinicians often fail to request tests for *C. difficile* toxins in cases of unexplained diarrhoea. In a Finnish study RCDI was associated with significantly longer length of stay (LOS) and higher costs compared to the average CDI population (Agthe et al 2012). The main cost driver between the groups was LOS (Figure 2, Agthe et al. 2012).

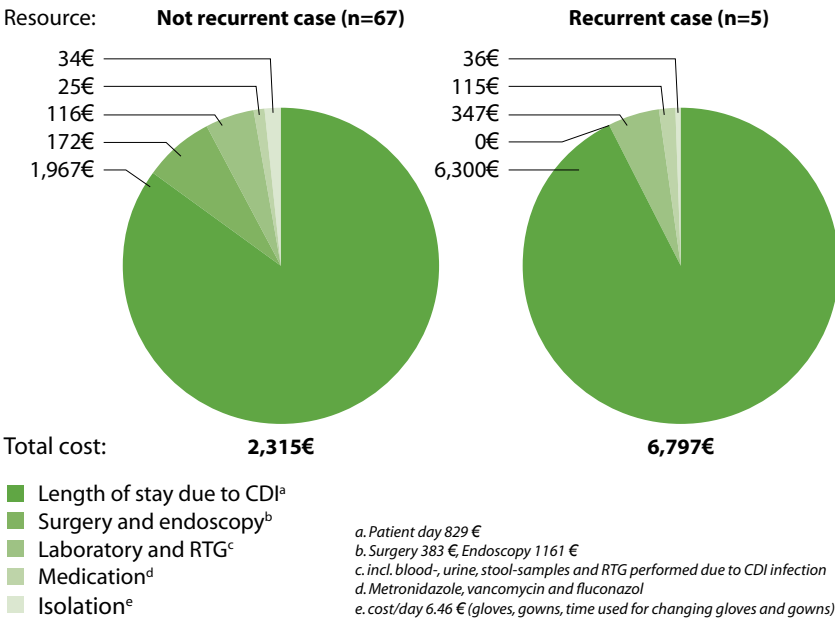


Figure 2 Healthcare costs for recurrent and not recurrent case (average/patient)

Data from the European survey showed that the overall mortality rate was 22%, with CDI being directly responsible for c. 2% of all deaths and a contributor to death in a further 7% of cases (Bauer et al. 2011). The overall mortality rate at day 30 was similar, 23 %, in a Canadian study (Pepin et al. 2005³).

2.3.4 CDI IN SPECIAL POPULATIONS

Children

As among adults, the epidemiology of CDI in children has been changing over the past decade. Historically, health care–associated diarrhea among children was attributed to viral pathogens (Langley et al 2002). Kim and colleagues showed a steady increase in the annual incidence of CDI among paediatric inpatients, from 2.6 to 4 cases per 1000 admissions in 22 US hospitals over the 5-year period to 2006 (Kim et al 2006). Also rates of pediatric CDI-related hospitalizations increased substantially between 1997 and 2006, from 0.724 to 1.28 per 1000 hospitalizations (Zillberg et al 2010). The increase was mainly due to high rates among children aged 1-4 years and non-newborns less than a year old. Colonisation with *C. difficile* in children appears to occur soon after birth and rises to very high levels (70%) at one year (Al-Jumaili et al. 1984), with high carriage rates particularly associated with hospitalisation (Enocha et al. 2011). Because of this it is difficult to determine whether CDI-related hospitalizations in this age group represent true infection or colonization. Asymptomatic carriage diminishes with age as the lower intestinal microbiota becomes established, usually by age of 2 years (Hafiz and Oakely 2012). In the 24–36-month age group, colonization was 6 % (Rousseau et al. 2012), a value close to that observed in adults. In the same study toxigenic clones were found during several months, in contrast with the succession of clones found in infants colonized by nontoxigenic strains. Symptomatic CDI appears to be strongly linked to the presence of co-morbidities such as haematological malignancies, immunosuppression and bowel disorders (Sandora et al. 2011, Tai et al. 2011). Reported rates of recurrence among children have been similar to those in adults (Kim et al. 2012).

Recently in a large multicenter retrospective analysis children who develop CDI after admission to the hospital have a >6-fold higher mortality rates than do controls with similar underlying disease and risk factors (Sammons et al 2013). In addition, children with CDI have significantly longer LOS and incur more total hospital costs than matched controls. Contact with infants aged <2 years has been linked with CDI in adults (14% versus 2%; $P < 0.02$) with community acquired disease (Wilcox et al. 2008). Still, the significance and outcomes associated with CDI, as well as the optimal diagnosis and treatment, remain poorly defined among children.

Peripartum Women

The estimated CDI incidence among peripartum women increased from 0.4 to 0.7 per 100,000 deliveries in USA from 1998 to 2006 (Kuntz et al. 2010). 67 % of CDI cases were observed in women who underwent Cesarean section. Women undergoing a Cesarean section tend to have significantly longer hospital stays than do women undergoing a vaginal delivery, placing them at increased risk of exposure to a nosocomial CDI. Most of these women had a history of recent antibiotic use. Cesarean section may be a particular risk for CDI that develops in the postpartum period (Unger et al. 2011, Venugopal et al. 2011).

Inflammatory bowel disease (IBD)

Similar to rising rates of CDI in the general population, patients with IBD (Crohn's disease and ulcerative colitis) have an increased incidence of CDI (Rodemann et al. 2007). In one study the majority of patients with IBD acquired CDI as outpatients (76 %) (Issa et al. 2008). CDI patients with IBD tend to have more severe disease, and are more likely to die or to need urgent colectomy than CDI patients who do not have underlying IBD (Ananthakrishnan and Binion 2010). Risk factors for CDI include severe underlying IBD, ongoing immunosuppression and colonic disease; thus rates of CDI are higher in individuals with ulcerative colitis than in those with Crohn's disease (Issa et al. 2008, Rodeman et al. 2007, Jodorkovsky et al. 2010, Jen et al. 2011). Among the different therapies, the highest risk appears to be with corticosteroid use, which confer a threefold increase of CDI. Corticosteroid exposure within 2 weeks of the diagnosis of CDI was also associated with a twofold increase in mortality (Das et al. 2010).

The clinical presentation of CDI and flare of IBD may be similar and requires a high index of suspicion for prompt detection and institution of appropriate therapy. Up to 20% of IBD flares are associated with a positive *C. difficile* stool result (Meyer et al. 2004), suggesting that CDI may not only mimic but can also precipitate an IBD flare. The American College of Gastroenterology (ACG) 2013 guidelines (Surawicz et al. 2013) recommend that all patients who require hospitalization because of an IBD flare, as well as ambulatory patients with risk factors for CDI (e.g., recent hospitalization, antibiotic use) or unexplained worsening of symptoms in the setting of previously quiescent disease, should be tested for *C. difficile*. The same guidelines recommend also ongoing immunosuppression be continued at existing doses in IBD-CDI patients. Escalation of the corticosteroid dose or initiation of anti-TNF (anti-tumour necrosis factor) therapy in patients with a positive CDI probably should be avoided for 72 hours after initiating therapy for CDI.

2.4 PATHOGENIC FEATURES OF *C. DIFFICILE*

2.4.1 PATHOGENESIS

C. difficile is an anaerobic, gram-positive, spore forming bacillus that is acquired via the fecal-oral route. *C. difficile* spores are the transmissible form, contribute to survival of the organism in the host, and are responsible for recurrence of disease when therapy is ceased. Like other bacterial spores, they are metabolically dormant and are resistant to desiccation, chemicals and extreme temperatures. Spores frequently contaminate the environment around patients with CDI, potentially persisting for months and even years. Although colonization of healthy non-hospitalized adults is uncommon (i.e., rate <5%), colonization among hospitalized patients and especially nursing home residents may range from 25% to 55% (Clabots et al 1992, Riggs et al 2007). Transmission in health-care facilities results mostly from environmental surface contamination and hand carriage by staff members and infected patients. Whereas vegetative cells are killed in the acidic environment of the stomach, acid resistant spores pass through relatively undamaged and convert to vegetative forms in the small bowel after exposure to primary bile acid (Wilson 1993).

Perturbation of the normal bowel microflora, most often from antibiotic use, leads to the loss of colonization resistance. In this setting *C. difficile* endogenous or exogenous spores germinate and vegetative cells multiply. The organism adheres to the mucus layer by means of its multiple adhesins and penetrates the mucus with aid of flagella and proteases. Once it penetrates mucus, the organism adheres to enterocytes and colonization begins.

Only toxigenic strains are associated with the development of *C. difficile* diarrhea. In summary the pathogenesis of CDI consist of alteration of the normal fecal flora, colonization with toxigenic *C. difficile* and growth of the organism with elaboration of its toxins

2.4.2 VIRULENCE FACTORS

Toxins

The main *C. difficile* virulence factors are the two large clostridial toxins, toxin A and toxin B. Toxin A and toxin B are encoded on the pathogenicity locus (PaLoc), which comprises five genes, *tcdA*, *tcdB*, *tcdC*, *tcdR*, and *tcdE*. Toxin A and toxin B are encoded by the genes *tcdA* and *tcdB*. *TcdR* gene and *tcdC* gene encode proteins involved in regulating the expression of toxin A and toxin B. The product of *tcdE* is postulated to facilitate the secretion of the toxins from the cell. The

DNA sequence of the PaLoc is variable, and strains with changes in this region are defined as different toxinotypes (Rupnik 2008). Nontoxigenic strains lack the PaLoc.

Some strains produce a third toxin known as binary toxin or *C. difficile* transferase (CDT). The clinical significance of binary toxin in CDI remains uncertain. It is found in approximately 6%–12.5% of strains overall (Carroll et al 2012). In a recent study patients with binary toxin had a higher 30-day case-fatality rates than patients without binary toxin, irrespective of PCR ribotype (Bacci et al 2011).

Sporulation and germination

It has also been postulated that increased sporulation may be associated with hypervirulence (Merrigan et al 2010, Dawson et al 2011) although this also remains controversial, particularly as in vitro experiments may not reflect in vivo behaviour. There is no simple relationship between antibiotic mediated depletion of the colonic microbiota and the induction of *C. difficile* spore germination with subsequent toxin production. Rather, antibiotic exposure might directly stimulate germination of spores and toxin production (Saxton et al 2009). The bacteriological response to vancomycin varies among strains and possibly correlates with the germination capacity (Baines et al 2008). Further investigation of the factors that affect both sporulation and germination could provide insights into the risk factors and treatment options for CDI.

Surface layer proteins and adherence

Surface proteins are integral to the adherence of the organism to the gut mucosa and can induce both inflammatory and antibody responses in the host (Calabi et al. 2002, Drudy et al 2004, Wright et al. 2005, Péchiné et al. 2005, Ausiello et al. 2006). There is considerable variability between the surface proteins of different strains. The precise role of these factors in the virulence of *C. difficile* is unclear. These surface-exposed proteins are potential candidates for vaccine targets and novel diagnostic tests.

Toxin variant strains, ribotype 027

Toxinotype refers to a particular strain of *C. difficile* based on polymerase chain reaction (PCR)-restriction fragment analysis of the PaLoc. All strains in a given toxinotype have identical changes in the PaLoc.

The variant toxin genes encode variant toxins with alterations in their substrate specificity or can even result in the absence of one or both toxins. In addition to

changes in *tcdA* and *tcdB*, changes in the other genes of the PaLoc may also alter virulence. Currently there are over 30 known toxinotypes (Carter et al. 2012).

It was initially believed that toxin A was the most important toxin in CDI, but recently the importance of toxin B has been re-stated. Most disease is caused by strains that produce both toxins, but 2% to 5% of disease is the result of only toxin B (Digg and Surawicz 2009). TcdA–TcdB+ strains can cause the entire spectrum of symptoms of CDI. Toxin B may also have the capacity to cause systemic damage to the host in addition to localised damage within the gut (Hamm et al. 2006). Multiple organ failure encountered with small percentage of patients may be a result of systemic toxin damage (Dobson et al 2003).

At the clinical level, given toxinotypes can be linked to specific disease characteristics or patient populations in epidemic settings, but in general, toxinotype is not predictive of clinical disease expression. It is likely that multiple factors determine whether a strain is virulent and/or epidemic. Hypervirulent refers to toxin variant strains of *C. difficile* that are associated with increased toxin production and severe clinical disease.

Many epidemics of CDI are caused by a novel strain, ribotype 027, which has unique characteristics that may explain the virulence. This strain produces a binary toxin and has a partial deletion in a toxin regulator gene (*tcdC*) that cause hyperproduction of toxins A and B *in vitro* (Akerlund et al. 2008, Warny et al. 2005). Aside from having altered TcdC, epidemic 027 strains have five unique genetic regions not present in historical 027 strains (Stabler et al. 2006). These genes include mutations that explain enhanced toxicity, motility, survival and increased sporulation. Also unlike historic isolates, epidemic isolates of *C difficile* ribotype 027 were resistant to fluoroquinolones (McDonald et al. 2005, Loo et al. 2005), which suggests that the increased use of quinolones may have influenced the emergence of this strain. Compared with other strains, ribotype 027 has a higher infection-to-colonization ratio (Loo et al. 2011) and it has been associated with a poorer response to therapy and higher recurrence rate (Huttunen et al. 2012), an effect observed across treatment types and despite lack of demonstrable resistance *in vitro* (Petrella et al. 2012).

2.5 RISK FACTORS OF CDI

2.5.1 RISK FACTORS FOR DISEASE

The most common risk factor for the development of CDI is recent or current antibiotic use, which leads to alteration in bowel microflora and the loss of colonization resistance. Other important risk factors include age greater than 65 years, multiple underlying comorbidities and hospitalization (McFarland 1998, Pepin et al. 2005²).

Almost all antimicrobial agents except for aminoglycosides have been associated with development of CDI (Suneshine and McDonald 2006). The risk is increased if *C. difficile* is resistant to the antimicrobial agents used (Johnson et al. 1999). Alternatively, antimicrobials that are active against *C. difficile* decrease the risk of colonization and infection during their use (Donskey 2004, Gerding 2004). The antibiotic susceptibility of *C. difficile* strains, including epidemic clones, is changing and it also varies widely between countries (Huangh et al. 2010). Even very limited exposure, such as single-dose surgical antibiotic prophylaxis, increases a patient's risk of both *C. difficile* colonization (Privitera et al. 1991) and symptomatic disease (Yee et al. 1991). In studies that evaluate risk for CDI after the use of an individual antimicrobial, treatment with multiple antimicrobials can lead to controversial results, making determination of risk inherently more difficult (Wilcox 2001). Therefore, it is difficult to assess the independent role of each antimicrobial to the risk of developing CDI. In general the number of administered antibiotics, their dosage and the duration of therapy have been identified as factors determining the risk for CDI (Owens et al. 2008, Dubberke et al. 2007, Wiström et al. 2001). CDI risk is elevated 7- to 10- fold during antibiotic therapy and the first month after cessation of antibiotics. It remains elevated for at least 3 months after administration of antibiotics (Hensgens et al. 2012¹).

The highest risk of developing CDI has been associated with use of clindamycin, cephalosporins, and fluoroquinolones (Table 3). At the moment, cephalosporins are the leading antimicrobial class associated with CDI (Muto et al. 2007, McCusker et al 2003, Loo et al. 2011, Owens et al. 2007). *C. difficile* isolates are fully resistant to most cephalosporins (Gerding 2004, Johnson et al.1999). The emergence and spread of *C. difficile* O27 correlates with acquired resistance to the fluoroquinolones, a trait that was not present in historic strains of the same genotype (Pepin et al. 2005², Muto et al. 2005, McCusker et al. 2003). Historically most of the prevalent types in human populations were clindamycin resistant (Labbe et al. 2008, Johnson et al. 1999). At the moment, *C. difficile* resistance for clindamycin is variable (Johnson et al. 2009). Clindamycin's relatively unique preference for impacting the intestinal flora over a prolonged period may increase the window

of susceptibility to CDI to a time point after the antimicrobial is discontinued (Sambol et al. 2002, Larson and Borriello 1990).

Table 2 Antimicrobial Agents Associated with CDI (those available in Finland).

Most frequently	Less frequently	Rarely or never
Ampicillin and amoxicillin	Carbapenems	Daptomycin
Cephalosporins	Other penicillins	Metronidazole
Clindamycin	Trimethoprim/sulfamethoxazole	Parenteral aminoglycosides
Fluoroquinolones		Rifampicin
		Tetracyclines, Tigecycline
		Vancomycin

Adapted from Higa and Kelly 2013 and Brown et al. 2013

There have been some recent reports of patients with community-onset CDI who had not been exposed to antibiotics. However, these cases are infrequent compared with the number of patients with CDI in hospital who have been exposed to an antibiotic in the 2–3 months before infection (Dial et al. 2008). Risk factors in the community are likely to be different. These may include genetically determined differences in immune reactivity and inherent differences in the ability of a specific individuals intestinal microbiome (Arungam et al. 2011) to resist colonization by *C. difficile*.

As a lower acidity environment allows vegetative forms of *C. difficile* to survive, emerging data indicate the need to avoid unnecessary use of gastric acid antisecretory medications. Two recent meta-analyses confirm association and strengthen the evidence that proton-pump inhibitor use is associated with an increased risk of CDI (Janarthanan et al 2012, Kwok et al 2012).

In addition, host factors play a role in the CDI development (Johnson et al. 2009, Gould and McDonald 2008). Colonization with *C. difficile* and high levels of serum IgG against *C. difficile* toxin A appear to provide protection against CDI (Shim et al. 1998, Kyne et al. 2000). Thus, the inability to mount an appropriate immune response in patients on chemotherapy or with severe underlying illness may explain the increased risk in these populations. Other important host factors are IBD (Rodeman et al. 2007, Issa, et al. 2008), use of feeding tubes or gastrointestinal surgery (McFarland et al. 1998, Bartlett et al. 1990). Variability in host factors may explain the wide spectrum of symptoms and course of disease.

2.5.2 RISK FACTORS FOR RECURRENT CDI

The risk factors for RCDI are slightly different from those for initial CDI. Two likely mechanistic factors increasing the risk of RCDI are an inadequate immune response to *C. difficile* toxins (Kyne et al. 2001) and decreased overall diversity of the gut microbiota (Chang et al. 2008). Important epidemiologic risk factors include advanced age over 65 years (Pepin et al. 2005¹, Bauer et al. 2011), continuation of other antibiotics, and prolonged hospital stays (Johnson et al. 2009, Pepin et al 2005¹, Eyre et al 2012). Infection with the 027 strain of *C. difficile* may also convey an increased risk of recurrence (Petrella et al. 2012), although this has not been confirmed in all recent analyses of risk factors for CDI recurrence (Eyre et al. 2012).

Patients are also at increased risk of recurrent CDI if they have severe or extremely severe underlying disease, as indicated by a modified Horn index (Table 3) score of 3 or 4 (Hu et al. 2009). In a recent study lymphopenia at the end of CDI treatment appeared to be a marker for CDI recurrence (Lavergne et al 2013). Once patients have experienced one recurrence of CDI, they are at significantly increased risk of further recurrences (Bauer et al. 2011). Also antibiotics used in *C. difficile* treatment alter the colonic microflora and therefore predispose to recurrence. The risk of recurrence more than doubles after two or more recurrences (McFarland 1998). Specific comorbidities that have been found to be associated with an increased risk of recurrent CDI include a compromised immune system (Cohen 2009), renal impairment (Do et al 1998) and inflammatory bowel disease (Kelsen et al. 2011).

Prominent risk factors have been examined to develop and validate a clinical prediction tool for recurrent CDI, with three factors (age >65 years, severe underlying disease (by the Horn index score, Table 3), and continued use of antibiotics for non-CDI infections) being highly predictive of CDI recurrence (Kelly 2013). Each predictor—age over 65 years, Horn index 3-4, and additional antibiotic use—was assigned 1 point. Patients with scores 2 or 3 were classified as high risk. The clinical prediction rule effectively discriminated between patients with and without recurrent CDI, with 77 % accuracy (Hu et al. 2009).

Table 3 Horns index of disease severity

	Score
Single mild illness	1
More severe illness but uncomplicated recovery expected	2
Major illness or complications or multiple conditions requiring treatment	3
Catastrophic illness that may lead to death	4

2.5.3 RISK FACTORS FOR ADVERSE OUTCOME

Of several clinical factors which have been linked to CDI of increased severity, and to adverse clinical sequelae, older age again has emerged as an important risk factor (Loo et al. 2011, Cohen et al. 2010). Factors that are strongly suggestive of severe CDI include an elevated peripheral white blood cell count ($>15 \times 10^9/l$), with counts above $50 \times 10^9/l$ being considered a warning of likely death (Lamontagne et al. 2007) and a rising serum creatinine level (Pepin et al. 2005³). Leukocytosis likely reflects the severity of colonic inflammation. An elevated serum creatinine level may indicate severe diarrhea with subsequent dehydration or inadequate renal perfusion (Loo et al. 2011). In addition pre-existing corticosteroid use is a potentially useful risk markers for mortality in CDI (Bloomfield et al. 2012). Fever, haematocrit, diarrhoea severity and several comorbidities were not associated with mortality in the meta-analysis, raising questions about their inclusion in CDI severity scores.

The role of particular ribotypes in the clinical outcome of CDI is complex (Bloomfield et al. 2012). *C. difficile* 027 has been suggested to cause a more severe disease than other ribotypes (Warny et al. 2005). However, recent report suggest that ribotypes 027 or 078 are not independent predictors of severe outcome when adjusted by the patient's leukocyte count and albumin level (Walk et al. 2012).

2.6 CLINICAL FEATURES OF CDI

The most common clinical presentation of CDI is diarrhea associated with a history of antibiotic use. Factors other than antimicrobial use that can predispose to CDI include bowel ischemia, recent bowel surgery, uremia, malnutrition and chemotherapy. The incubation period from ingestion of *C. difficile* to onset of symptoms has been estimated to be a median of 2–3 days (McFarland et al 1989, Johnson et al 1990, Samore et al 1994). In some patients, no recent antibiotic use or health care exposures are identified. Colonization and infection with toxigenic strains can lead to a spectrum of illness including asymptomatic carriage, or mild diarrhea, which resolves with discontinuation of antibiotics, to fulminant colitis, which has high mortality. The onset of diarrhea is typically during or shortly after receipt of a course of antibiotic therapy but may occur from a few days after the initiation of antibiotic therapy to as long as 8 weeks after the termination of therapy (Mogg et al. 1979).

In some patients disease is localized to the proximal colon. These patients may present with an acute abdomen, localized rebound tenderness and no diarrhea. Considering this diagnosis in such a patient with subsequent confirmation based on stool studies and computed tomography (CT) may help avoid unnecessary surgery

(Drapkin et al. 1985). Overall, fever occurs in 28% of cases, leukocytosis in 50%, and abdominal pain in 22% (Bartlett et al. 1980).

2.6.1 ASYMPTOMATIC CARRIAGE

Carriage of *C. difficile* occurs in 5 – 15 % of healthy adults and may be transient (Ozaki, et al. 2005, Matsuki et al.2005, Nakamura et al. 1981). Among the elderly, carriage rates may be higher especially in those in long-term care or nursing home facility. Several studies have alluded to the importance of asymptomatic *C. difficile* carriers as a potential source of transmission (Riggs et al. 2007, Sethi et al. 2010, Johnson et al.1990). In a study of elderly patients in a long-term care facility affected by an outbreak of CDI, asymptomatic carriers outnumbered symptomatic patients by seven to one (Sethi et al 2010). However, levels of *C. difficile* contamination on the skin and in the surrounding environment of carriers approached those for symptomatic patients, suggesting that the former may be an important source of onward transmission (Sethi et al. 2010). In this respect, it is noteworthy that many CDI patients in whom diarrhoea resolves following a course of specific antibiotic therapy become asymptomatic carriers, and may continue shedding *C. difficile* spores for several weeks after treatment has ended.

2.6.2 MILD TO MODERATE CDI

Mild disease consists of mild to moderate nonbloody diarrhea with minimal systemic symptoms and a normal physical examination. Diarrhea is usually the only symptom, with patients experiencing up to but usually considerably less than 10 bowel movements per day (Bartlett 2002). Stools are usually watery, with a characteristic foul odour, although mucous or soft stools also occur. Patients can also present with symptoms of colitis: fever and lower abdominal cramps.

2.6.3 SEVERE CDI

Around 10% of cases of CDI have clinical features consistent with severe CDI (Muto et al 2005). There is no universally agreed upon definition for severe CDI. The Society for Healthcare Epidemiology of America/Infectious Diseases Society of America (SHEA/IDSA) guidelines define severe CDI on the basis of WBC greater than 15,000/L or a level of creatinine 1.5-fold above the patient's baseline value (Cohen et al 2010). Severe disease is characterized by profuse, usually non

bloody, diarrhea, abdominal pain, fever, nausea, anorexia, malaise, and abdominal tenderness. C-reactive protein and leukocytes can be moderately or even highly elevated, and a leukemoid reaction is not a rarity. In one study 58 % of the patients with unexplained leukocytosis had CDI (Wanahita et al 2003). Hypoalbuminemia is also a common feature because CDI is a protein-losing enteropathy and low albumin is considered a marker of inflammatory states.

2.6.4 FULMINANT CDI

Fulminant colitis occurs among 1%–3% of patients and is characterized by signs and symptoms of severe toxicity with fever, and diffuse abdominal pain and distension (Triadafilopoulos and Hallstone 1991, Rubin et al. 1995, Kelly et al. 1994). The timing from onset of any CDI symptoms to fulminate colitis varies from weeks to just a couple of hours; patients with rapid progression have worse outcomes. (Dallal et al. 2002). Although profuse diarrhea may be present, patients with severe pseudomembranous colitis may have little to no diarrhea if they have an associated paralytic ileus or toxic megacolon (Kelly and LaMont 1998). Complications include colonic perforation and peritonitis. Abdominal images show air if colonic perforation has occurred and diffuse colonic inflammation. Colonoscopy reveals diffuse inflammation and possibly pseudomembranes. Pseudomembranes can exist throughout the entire colon, but they are usually most pronounced in the rectosigmoid colon.

Patients with a WBC of greater than $50 \times 10^9 / l$ or level of lactate greater than 5 mmol/L have a poor prognosis (Lamontagne et al 2007). Mortality associated with toxic megacolon is high, ranging from 24% to 38%. (Dobson et al. 2003, Lipsett et al. 1994, Morris et al. 1990).

2.6.5 RECURRENT CDI

Recurrent disease is defined as symptomatic CDI that recurs after completion of an appropriate course of antibiotics for the initial infection. Recurrence can be due to either relapse of infection caused by the original strain or re-infection caused by a different strain (Barbut et al. 2000, Johnson et al. 1989). In clinical practice, it is impossible to distinguish these mechanisms of recurrences. Lack of restoration of enteric microbiota, persistence of *C. difficile* spores within the gut and failure of the host to establish an adequate immune response to *C. difficile* toxins A and B (Johnson et al. 1989, Chang et al. 2008, Kyne et al. 2001) appear to all be related with the chance of recurrence.

Clinical severity and outcomes do not change significantly between primary infection and recurrences (Louie et al 2011). Recurrence typically happens within 14 days after cessation of antibiotic treatment for the initial episode; however, it can occur for up to 12 weeks after stopping antibiotics (Kelly 2009). The overall risk of RCDI has been reported as 10-20% after initial CDI (Surawicz et al. 2013), 45% after a first relapse, and greater than 60% for those with 2 or more recurrences (Bartlett 1990). Persisting diarrhea after resolution of CDI may not be caused by a recurrence but instead may reflect simple AAD or be a form of postinfectious irritable bowel syndrome (IBS). In one recent study persistent diarrhea in CDI correlated with intestinal inflammation markers and not fecal CDI burden (El Feghaly et al. 2013).

2.6.6 DIFFERENTIAL DIAGNOSIS OF CDI

None of these clinical features are specific to CDI, and a variety of disorders may cause similar clinical presentations. These include diarrhea caused by other enteric pathogens, AAD, inflammatory bowel disease, adverse reactions to other medications, ischemic colitis and intra-abdominal sepsis. The presence of fever and leukocytosis favour *C. difficile* or other infectious etiology. Postinfectious IBS occurs in about 10 % of patients after successful CDI treatment (Kelly 2009). These patients may have watery diarrhoea mimicking CDI.

Antibiotic-associated haemorrhagic colitis (AAHC) is an uncommon cause of bloody diarrhoea in patients taking penicillin or penicillin-related antibiotics. (Moulis and Vender 1994, Toffler et al. 1978, de Mulder 1978, Barrison and Kane 1978). It has also been reported after antibiotic therapy with quinolones and cephalosporins (Koga et al. 1999). The accumulated evidence implicates *Klebsiella oxytoca* as a probable cause of AAHC (Beaugerie et al 2003, Benoit et al. 1992, Högenauer et al. 2006). Some *K. oxytoca* strains isolated from patients with AAHC produce a cytotoxin that can induce epithelial cell death and may predispose certain patients to hemorrhagic colitis during exposure to antibiotics. *K. oxytoca* also produces a chromosomally encoded beta-lactamase that renders it resistant to aminopenicillins. Therapy with these antibiotics and others to which *K. oxytoca* is resistant presumably contributes to its overgrowth and the development of AAHC. Other possible mechanisms for AAHC include allergic reaction (Toffler et al. 1978) and mucosal ischemia (Yonei et al. 1996).

Characteristically, the symptoms of AAHC begin within 2-7 days of antibiotic use. Patients develop sudden onset of lower abdominal pain and loose, watery stools, followed within 6 hours by rectal blood loss (Moulis and Vender 1994). Its rapid resolution after cessation of antibiotics (Sakurai et al. 1994) and its predilection for the right side of the colon (Iida et al. 1985) may result in the

diagnosis being missed if a full colonoscopy is not performed within days of the onset of symptoms. The key macroscopic feature is segmental distribution of mucosal hemorrhage and mucosal edema localized predominantly in the right colon, with lack of pseudomembranes.

2.6.7 EXTRAINTESTINAL CDI

Extraintestinal manifestations of CDI are unusual. There are case reports of rare presentations of CDI, including patients with bacteremia, intra-abdominal and perianal abscesses, peritonitis, wound and joint infections (Feldman et al. 1995, Byl et al. 1996, Wolf et al. 1998, Deptula et al 2009). Extraintestinal infections with *C. difficile* are often polymicrobial and identified among patients with underlying comorbid conditions (Wolf et al 1998).

Reactive or postinfectious syndromes can occur after CDI, including reactive arthritis and IBS (Birnbaum et al 2008, Sethi et al 2011). As with other reactive arthritides after enteric infections, many of these patients are positive for the HLA-B27 (Atkinson and McLeod 1998, Hayward et al 1990).

2.7 DIAGNOSIS OF CDI

2.7.1 LABORATORY DIAGNOSIS

The best standard laboratory test for diagnosis has not been clearly established. For the past 30 years, the two primary reference tests are the *C. difficile* cytotoxin neutralization assay (CCNA) and toxigenic culture (TC) (Planche and Wilcox 2011, Sambol et al. 2000). The first detects the presence of *C. difficile* toxins, toxin B and toxin A, in a fecal sample. By contrast, TC detects *C. difficile* strains that have the capacity to produce toxins. *C. difficile* culture alone is not sufficient because not all *C. difficile* strains produce toxin (Rea et al. 2012, Viscidi et al. 1981). The enzyme immunoassay (EIA) for detection of toxins A and B has been the most widely used diagnostic test for CDI because of its rapid turnaround, low cost, and simplicity. However, EIAs for toxins A and B are known to have low sensitivity (60%–80%) compared with TC (Eastwood et al 2009). Different strains of *C. difficile* can provide different results in toxin EIA assays (Tenover et al 2010).

C. difficile nucleic acid amplification test identifies genes (not the toxin) that encode the toxins (usually toxin B) by using PCR or loop-mediated isothermal

amplification of DNA. These assays have a short turnaround time, and their sensitivities range from 84% to 94% compared with toxigenic stool culture, making their use by clinical laboratories very attractive (Deshpande et al 2011). The use of more sensitive and rapid testing for CDI diagnosis may lead to treatment of patients before they progress to severe CDI. However, healthcare facilities should expect an increase in CDI rates when transitioning to a PCR-based assay and also should emphasize appropriate testing practices to avoid detection of asymptomatic carriers. As diarrhea is a common symptom in the hospitalized, elderly or long term care facility patient, it remains difficult to distinguish the patient with CDI from the patient for whom a positive *C. difficile* test is related to underlying colonization. Finally, all laboratory tests must be interpreted in the context of patient symptoms and risk factors for CDI.

Only stools from patients with diarrhea should be tested for *C. difficile*. (Cohen et al. 2010). Very occasionally, a patient with ileus and complicated disease will have a formed stool. Rectal swabs can be used for PCR and thus may be useful in timely diagnosis of patients with ileus (Kundrapu et al 2012). Several studies have shown that repeat testing after a negative test is positive in < 5 % of specimens and repeat testing increases the likelihood of false positives (Debast et al. 2008, Deshpande et al. 2010, Luo and Banei 2010). If repeat testing is requested, the physician should confer with the laboratory to explain the clinical rationale. Both toxin A + B EIA and TC may remain positive for as long as 30 days in patients who have resolution of symptoms (Surawicz et al. 2000, Wenisch et al. 1996). False positive “ test of cure ” specimens may complicate clinical care and result in additional courses of inappropriate anti- *C. difficile* therapy.

2.7.2 ROLE OF ENDOSCOPY AND RADIOLOGY

C. difficile most often causes a nonspecific colitis. However, especially in more severe cases, one may see the distinct macroscopic appearance of pseudomembranous colitis at endoscopy or by histopathologic examination. At least 90 % of patients with pseudomembranous colitis demonstrate either *C. difficile* or its toxins in stool samples (Wolfhagen et al. 1994). Many milder cases only reveal the nonspecific findings of erythema and edema. Notably, patients with IBD may not exhibit pseudomembranes or classic histologic findings at all (Issa et al 2008). Endoscopy provides the added benefit of aiding in the identification of other causes of diarrhea, such as cytomegalovirus colitis, graft-versus-host disease or, in the case of bloody diarrhea, ischemic colitis or IBD.

Computed tomography (CT) scan is normally not required for diagnosing CDI, especially for mild-to-moderate disease, but it can be useful for recognizing

more severe forms. Colonic inflammation can also be shown on CT as increased thickening of colonic wall (Ash et al 2006) with trapping of contrast material, pancolitis, pericolonic fat changes and ascites

2.8 TREATMENT OF CDI

For 30 years, metronidazole and oral vancomycin have been the main antimicrobial agents used in the treatment of CDI. On the basis of two randomized controlled trials with oral vancomycin, the FDA and EMEA (European Medicine Agency) granted approval for a new macrocyclic antibiotic, fidaxomicin in 2011 (Louie et al. 2011, Cornely et al. 2012). In addition to antimicrobials, the prevention and treatment of CDI may include infection control measures, antimicrobial stewardship, restoration of the protective microbiota, and increased immunity to *C. difficile* toxins.

The first step in treatment is cessation of the inciting antibiotics, if this is deemed to be medically appropriate. Withdrawing the offending antibiotic will lead to resolution of CDI within 48 hours in up to 20% of the cases (Teasley et al. 1983). Treatment for CDI can be initiated before laboratory confirmation for patients with a high pre-test suspicion of disease. There is no basis for prophylactic antibiotics for patients at risk of CDI or asymptomatic colonization with *C. difficile* (Dubberke et al. 2008).

When administered orally, metronidazole is absorbed rapidly and almost completely in the small intestine and then excreted again in the bile and in the inflamed colon (Bolton and Culshaw 1986). Metronidazole is not present in stool samples of asymptomatic patients (Johnson et al. 1992). There may be low levels measured in the presence of diarrhea, but concentrations decrease rapidly after treatment of CDI is initiated. The activity of metronidazole against CDI depends on back diffusion from the serum across the colonic mucosa, but this is quite fluctuating (Bolton and Culshaw 1986). Given the relatively low fecal concentrations achieved with metronidazole, even a modest decrease in susceptibility might have a marked effect on treatment efficacy in CDI. Previous reports have uniformly demonstrated that metronidazole has very good *in vitro* activity against *C. difficile* (Shuttleworth et al. 1980). In one recent study minimum inhibitory concentration (MIC) obtained by Etest were lower compared with those obtained by agar dilution method (ADM) and agar incorporation method (AIM), causing discrepancies in the categorization (as susceptible or having reduced susceptibility) of some strains (Moura et al. 2013). In another study up to 24.% of the *C. difficile* ribotype 001 isolates demonstrated decreased susceptibility or resistance using the spiral gradient endpoint method and the AIM, but not using E-test.(Baines et al. 2008). Even if the significance of both *in vitro* resistance and heteroresistance (Pelaez et al. 2008) to metronidazole in the

treatment of CDI remains unclear, the low fecal concentrations of metronidazole suggests that *C. difficile* subpopulations with reduced susceptibility to this antibiotic may be one factor responsible for reduced metronidazole efficacy in vivo. Thus far the reduced clinical response to treatment with metronidazole has not been attributed to resistance to the drug in *C. difficile*.

Vancomycin has excellent *in vitro* activity against *C. difficile*, with a MIC of 0.75–2.00 µg/ml required to inhibit 90% of strains (Wong et al. 1999, Marchese et al. 2000, Jamal et al. 2002). One study has reported intermediate *in vitro* resistance to vancomycin in 3% of *C. difficile* isolates, with a MIC of 4–16 µg/ml required to inhibit the growth of these strains (Pelaez et al. 2002). Unlike metronidazole, vancomycin is poorly absorbed, and fecal concentrations following oral administration reach very high levels. Vancomycin levels in the colonic lumen are over 100-fold greater than the highest MIC ever measured for a strain of *C. difficile* (Bartlett 2009). So emergence of resistance is likely not a concern. Fecal levels achieved are high enough that organisms generally considered to be even vancomycin insensitive, such as the gram-negative *Bacteroides fragilis* group, can be affected both in vitro (Finegold et al. 2004) and in vivo (Louie et al. 2009). Given its poor absorption, orally administered vancomycin is relatively free of systemic toxicity. Since intravenous vancomycin is not able to reach the lumen of the colon, it has no role in the therapy of CDI. Emergence of vancomycin-resistant *Enterococcus* (VRE) has not been shown to be a valid reason to avoid use of vancomycin for treatment of CDI, as both vancomycin and metronidazole treatment for CDI have been shown to promote VRE acquisition in prospective observational studies (Al-Nassir et al. 2008).

Early prospective, randomized trials concluded that metronidazole was not inferior to vancomycin, with initial cure rates over 90% (Teasley et al. 1983, Wenisch et al. 1996). Decreased response rates and slower responses for metronidazole have been noted since 2004 (Fernandez et al. 2004, Musher et al. 2005, Pepin et al. 2007, Belmares et al. 2007, Lagrotteria et al. 2006). However, a 2007 Cochrane meta-analysis of 12 randomized trials showed that none of 8 antibiotics was superior in terms of outcome, and favored metronidazole as initial therapy for its lower cost and similar efficacy (Nelson 2007). In the same year, in a large prospective, randomized and blinded study vancomycin was shown to be superior to metronidazole in cases of severe CDI (Zar et al. 2007). Again, in 2011 published systematic review of the comparative effectiveness of CDI treatments, the three studies directly comparing vancomycin and metronidazole failed to show a significant difference between the two treatments (Drekonja et al. 2011). Limitations of the available evidence include substantial variability among studies, including the definitions used for CDI, initial cure and recurrence, and the durations of treatment and follow-up.

Fidaxomicin has minimum systemic absorption, high faecal concentrations and restricted activity against normal gut flora (Louie et al. 2009, Tannock et al. 2010). There is no evidence for cross-resistance between fidaxomicin and other classes of antibiotics. In vitro frequency of spontaneous mutations has been demonstrated to be low. In both published phase 3 trials (Louie et al. 2011, Cornely et al. 2012), fidaxomicin demonstrated non-inferiority to vancomycin for clinical response at the end of therapy and showed lower rates of relapse when compared to vancomycin in patients infected with non 027 *C. difficile* strains. There are limitations to these findings. Neither trial extended to 90 days and there is no biological plausibility to explain a strain-specific superiority of fidaxomicin. Additional literature suggests that fidaxomicin might have a favourable profile compared with alternate regimens when patients require additional concomitant antibiotics (Mullane et al. 2011). More study is needed to determine the place of fidaxomicin in treatment of patients with severe CDI and patients infected with the 027 strain of *C. difficile*.

Nitazoxanide, an antimicrobial agent already approved for *Giardia* and *Cryptosporidium* infections, has been shown to have statistically comparable efficacy with metronidazole in a small prospective randomized trial (Musher et al. 2009). It may also have a role in cases of CDI nonresponsive to metronidazole, although there are mixed data with comparison to vancomycin (Gerding and Johnson 2010). Larger studies comparing the efficacy of nitazoxanide with that of standard therapies are needed to define its place in the management of CDI and to test its noninferiority to currently available agents.

2.8.1 TREATMENT OF A FIRST EPISODE OF CDI

The treatment of CDI is described in Table 4. Treatment of CDI should be based on disease severity, although it is difficult to set a rigid set of criteria for the assessment of prognosis and severity of CDI. Patients with mild-to-moderate CDI should be treated with metronidazole 500 mg orally three times per day for 10 days. Patients with severe CDI should be treated with vancomycin 125 mg orally four times per day for 10 days. The assessment of disease severity can be made by evaluating clinical characteristics including fever, age, ICU admission, elevated WBC or creatinine, or low albumin (Zar et al. 2007).

Failure to respond to metronidazole therapy within 5-7 days should prompt consideration of a change in therapy to vancomycin at standard dosing. (Musher et al. 2005, Surawicz et al. 2013). The time to resolution of diarrhea might be shorter with vancomycin than with metronidazole therapy (Belmares et al. 2007). Prospective trials have not compared regimens with durations longer than 10 days. There is no evidence to support administration of combination therapy to patients

with uncomplicated CDI. The use of anti-peristaltic agents to control diarrhea from confirmed or suspected CDI should be limited or avoided, with concern for minimizing impaired toxin clearance and precipitate complicated disease (Koo et al. 2009, Kato et al. 2008).

In patients who are allergic or intolerant (e.g. nausea, vomiting, and taste disturbances) to metronidazole and for pregnant / breastfeeding women, vancomycin should be used. First trimester exposure to metronidazole is not recommended in FDA guidelines because of concern regarding ready placental transmission and possible facial anomalies following exposure (Surawicz et al. 2013).

Table 4 Treatment of CDI

Asymptomatic carrier	No treatment required
Initial CDI	Oral metronidazole 400mg t.i.d for 10 days
Severe CDI	Oral vancomycin 125mg four times a day for 10 days
Complicated CDI	Intravenous metronidazole 500mg t.i.d. and oral vancomycin 500 mg four times a day
1 st recurrence of CDI	Oral metronidazole 400mg t.i.d or oral vancomycin 125mg four times a day for 10 days
2 st recurrence of CDI	Vancomycin 125mg four times a day for 7 days and then tapering doses
3 st recurrence of CDI	FMT or oral fidaxomicin 200mg twice a day for 10 days or oral rifaximin 200mg four times a day for 10 days

Modified by Surawicz et al. 2013

2.8.2 TREATMENT OF SEVERE, COMPLICATED CDI

Severe complicated or fulminant CDI denotes progression to a complication like megacolon, ileus, or other sign of severe systemic involvement, including hypotension or any evidence of end organ failure and metabolic derangements including lactic acidosis (Higa and Kelly 2013). Symptoms of ileus include acute nausea, emesis, sudden cessation of diarrhea, abdominal distension, while radiological signs are consistent with disturbed intestinal transit. First-line therapy is oral vancomycin at a dose of 500 mg every 6 hours for 10 to 14 days, with the addition of intravenous metronidazole 500 mg every 6 hours. Direct instillation of vancomycin via colonic retention enema, colonoscopy, or long rectal tube is recommended if ileus is present (Shen and Surawicz 2008). For this approach, vancomycin 500 mg in a volume of at least 500 ml four times per

day is recommended (Olson et al. 2004, Pasic et al. 1993, Apisarnthanarak et al. 2002). Use of high doses of colonic administration of vancomycin is safe, but high serum concentrations have been noted with long courses of 2 g per day, with renal failure. It would be appropriate to obtain trough serum concentrations in this circumstance. The use of empiric antibiotics (other than those used to treat CDI) should be minimized and limited to situations where there is a clear indication (Higa and Kelly 2013).

Passive immunotherapy with intravenous immunoglobulins has been used for some patients not responding to other therapies (McPherson et al 2006) but no controlled trials have been performed. Case reports have suggested that tigecycline may be successful for treatment of severe or severe complicated CDI, when prior therapy has failed (El-Herte et al. 2012, Herpers et al. 2009, Lu et al. 2010). Tigecycline is a derivative of minocycline and it is administered intravenously. Tigecycline achieves fecal concentrations well above the MIC for *C. difficile*, because of primary biliary excretion of unchanged drug.

2.8.3 SURGERY FOR COMPLICATED CDI

Emergent colectomy can be life saving in severe disease. No randomized trials exist of surgical management of fulminant CDI. Indications for colectomy include toxic megacolon, perforation, peritonitis, severe complicated disease including shock/organ failure, and disease persistence or progression despite appropriate medical therapy (Lamontagne et al 2007). Currently, there is no scoring system that creates a threshold for operative management. ACG guidelines recommend consideration of surgical therapy in patients with any one of the following attributed to CDI: hypotension requiring vasopressor therapy; clinical signs of sepsis and organ dysfunction; mental status changes; WBC count $\geq 50,000$ cells/ μ l, lactate ≥ 5 mmol / l; or complicated CDI with failure to improve on medical therapy after 5 days (Surawicz et al. 2013). Fulminant CDI was indication for every third of colectomies in a Finnish tertiary-level mixed intensive care unit (Sipola et al. 2013).

The standard surgical approach is to perform a total colectomy with preservation of the rectum and a temporary end ileostomy. However, one recent case-controlled study treated patients with severe, complicated disease with loop ileostomy (Neal et al. 2011). Survival of patients compared with historical controls who had undergone colectomy improved (19 % vs. 50 %) with this new treatment. Other advantages are the potential preservation of the colon and fewer long-term adverse consequences.

2.8.4 TREATMENT OF RECURRENT CDI

Management of RCDI is poorly studied, and the recently published clinical practice guidelines give recommendations for RCDI that are based on relatively poor quality of evidence (Cohen et al. 2010, Surawicz et al. 2013, Bauer et al. 2009). RCDI is a therapeutic challenge because there is no uniformly effective therapy. Treatment of a first recurrence of CDI depends on the presentation at the time of recurrence and is stratified depending on disease severity in the same way as was for the treatment of an initial episode. Using either metronidazole or vancomycin treatment of a first recurrence does not alter the probability of a second recurrence (Pepin et al. 2006). Positive toxin assay at time of completion of therapy for CDI does not predict risk of relapse (Dubberke et al. 2007). Recurrence is not a result of antimicrobial resistance to metronidazole or vancomycin but rather an impairment of colonization resistance resulting from recent or continued antibiotic use or due to an impaired immune response.

In an observational study metronidazole was not inferior to vancomycin for treating patients with a first recurrence of CDI (Pepin et al. 2006). Metronidazole should not be used beyond the first recurrence or for long-term therapy because of peripheral neuropathy and other adverse effects (Kapoor et al. 1999). A substantial proportion of patients with a second recurrence will be cured with vancomycin with use of a taper and/or pulsed regimen. (McFarland et al. 2002). There are no controlled data to support specific tapering or pulse regimens (Tedesco et al. 1985). Options for treatment of a second or subsequent recurrence include a prolonged, tapering, and then pulsed dose oral vancomycin, oral fidaxomicin, or oral vancomycin followed by oral rifaximin (McFarland et al. 2002, Louie et al. 2011, Garey et al. 2011).

Rifaximin is a poorly absorbed oral rifamycin derivative, which has been proposed as a rescue option in the treatment of second and later recurrences. *C. difficile* usually shows good *in vitro* susceptibility to rifaximin, but MICs may rise postexposure which raises concerns of the potential for resistance to develop (Koo and DuPont 2010). Data from uncontrolled and relatively small studies suggest that rifaximin may have a role in the treatment of patients with multiple recurrences or those for whom other treatments have failed (Johnson et al. 2009, Garey et al. 2011).

2.8.5 TREATMENT OF THIRD AND SUBSEQUENT RELAPSES

In addition to tapered and pulsed vancomycin regimens, other management strategies for multiple CDI recurrences that have been reported in uncontrolled case series and appear to be useful include standard therapy with probiotics,

standard therapy followed by rifaximin, switching to nitazoxanide, intravenous immunoglobulin, and FMT (Johnson 2009).

2.8.6 FECAL MICROBIOTA TRANSPLANTATION

FMT is the term used when stool is taken from a healthy individual and instilled into a sick person to cure a certain disease (Bakken et al 2011). ACG guidelines recommend to consider FMT, if there is a third recurrence after a pulsed vancomycin regimen. An randomized controlled trial of donor feces administered by duodenal infusion with gut lavage showed significant efficacy compared to vancomycin or vancomycin with gut lavage without donor feces (van Nood et al 2013). The cure rate with FMT was 81 % compared to 23 % with vancomycin alone and 31% with vancomycin and gut lavage. Considering that disruption of the indigenous fecal flora is likely a major risk for infection with *C. difficile* and, particularly, for RCDI, instillation of stool from a healthy donor has been used with a high degree of success in several other uncontrolled case series (Kassam et al 2013).

Methods of administration of donor stool include by enema, whole bowel irrigation through a nasogastric tube, or colonoscopy. By 2011, approximately 325 cases of FMT had been reported worldwide, including approximately 75 % of them by colonoscopy or retention enema, and 25 % by nasogastric or nasoduodenal tube, or by esophagogastroduodenoscopy (Brandt and Reddy 2011, Gough et al 2011). Posttransplant evaluations show resurgence of native *Bacteroides* species frequently missing in the flora of those afflicted with CDI (Khoruts et al 2010). In one series, a standardized filtered, frozen, and then thawed preparation of stool from pre-screened universal donors showed cure rates equal to or better than those from patient-identified donors (Hamilton et al 2013).

FMT appears to be safe, with no adverse effects or complications directly attributed to the procedure yet described in the existing literature (Bakken et al 2011, Borody et al 2004).

The availability of this treatment is limited, however. If FMT is considered, the donor should be screened for transmissible agents, and logistic issues need to be considered, including the timing, the collection and processing of the specimen from the donor and the preparation of the recipient. Despite reported overall success rates of approximately 90%, this approach continues to be underutilized for aesthetic and logistical reasons (Yoon and Brandt 2010).

2.8.7 ROLE OF PROBIOTICS

Probiotics are living organisms that are beneficial to the host when given orally. Several individual trials using *Saccharomyces boulardii*, *Lactobacillus* species, or probiotic mixtures, always as an adjunct to antibiotics have indicated possible efficacy in preventing recurrent CDI. One uncontrolled study using Kefir (fermented milk drink made with kefir grains) as an adjunct to antibiotics did result in decreased recurrence of *C. difficile* (Bakken 2009). However, randomized-controlled trials have not demonstrated reproducible efficacy of probiotics in CDI prophylaxis or as primary treatment. (Na and Kelly 2011, Pillai and Nelson 2008). Thus, although probiotics demonstrate beneficial effects for other indications, they cannot be relied upon for prophylaxis against primary or recurrent CDI (Miller 2009). Probiotics are live organisms and treatment with probiotics is associated with risks, such as fungemia, bacteremia and endocarditis (Liong 2008). Probiotic *Saccharomyces boulardii* should not be given critically ill or immunocompromised patients (Enache-Angoulvant and Hennequin 2005).

2.8.8 IMMUNOTHERAPY

The only currently available immunotherapy for CDI is pooled intravenous immunoglobulin (IVIG). IVIG preparations contain neutralizing levels of IgG antibody to toxin A and toxin B (Salcedo et al. 1997). No conclusive evidence of benefit for IVIG has been demonstrated in retrospective analyses of its use for treatment of RCDI, nor has an effective dose been established (McPherson et al. 2006, Wilcox 2004). In a phase II clinical trial, a single infusion of monoclonal antibody to toxins A and B used as an adjuvant to standard antibiotic therapy significantly reduced the rates of RCDI compared to placebo (7% vs. 25%, $p < .001$) (Lowy et al. 2010). Additionally, this significant difference in recurrent rates was observed among patients with the *C. difficile* 027 strain and those with more than 1 prior episode of CDI.

An oral anti-*Clostridium* whey protein from cows immunized to *C. difficile* toxoid was studied in the Netherlands. Following successful studies in hamsters, the investigators found in an uncontrolled pilot study that anti-*Clostridium* whey protein was safe and well-tolerated in 16 patients with CDI (Young et al. 2007). None of the patients treated experienced recurrent CDI. Further development of this product has been halted due to lack of funding.

2.9 INFECTION PREVENTION AND CONTROL

The primary mode of *C. difficile* transmission is person-to-person spread through the fecal-oral route, mainly within inpatient healthcare facilities. Acquisition of *C. difficile* is facilitated by its ability to form spores. Spores may persist in the environment for months or years (Fekety et al. 1981) and they survive routine environmental cleaning with detergents and hand hygiene with alcohol-based gels.

Control measures of *C. difficile* are based on the prevention of cross-transmission, active surveillance of cases and prudent use of antimicrobial agents (Vonberg et al. 2008). Contaminated inanimate surfaces and transient hand carriage of health care workers and patients are important mediators for *C. difficile* transmission in hospitals (McFarland et al. 1989). Environmental disinfection, proper hand hygiene and the use of barrier methods are key components in preventing *C. difficile* transmission. Enhanced cleaning of all potentially contaminated surfaces with 10% sodium hypochlorite reduces the environmental burden of *C. difficile*. Hand washing with chlorhexidine or with soap and water has been shown to be effective in removing *C. difficile* spores from hands (Gerding et al. 2008). Other barrier precautions include use of gloves, gowns and contact precaution signs during patient contact.

Contact precautions for a patient with CDI should be maintained at a minimum until the resolution of diarrhea. One recommendation is to maintain contact precautions for 48 h after diarrhea ceases (Vonberg et al. 2008) or the whole duration of hospitalization (Muto et al. 2007). Prompt identification of patients with symptomatic *C. difficile* infection is essential to implement isolation precautions and treatment and to decrease the environmental bioburden associated with diarrhea.

Good antimicrobial stewardship complements infection control efforts and environmental interventions. A significant decrease in CDI rates was shown in a study by changing the class of antibiotics used as one-time doses for surgical prophylaxis (Al-Obaydi et al. 2010). Antimicrobials to be targeted ideally should be based on the local epidemiology and the *C. difficile* strains present, but restricting the use of the clindamycin and cephalosporins have been the most effective. The results of fluoroquinolone restriction have been variable but may be of particular importance of outbreaks associated with the fluoroquinolone resistant strains (e.g. 027). The efficacy of metronidazole or vancomycin prophylaxis to prevent CDI in patients who are receiving other antimicrobials is unproven, and treatment with these agents is ineffective against *C. difficile* in asymptomatic carriers (Gerding et al. 2008). Several guidelines for antibiotic stewardship programs have been published (Dellit et al. 2007, Lucado et al. 2012, Davey et al. 2005).

CDI outbreaks are often multifactorial in terms of cause. One observational study issued a “bundle” on CDI prevention, consisting of education, increased and early case finding, expanded infection control measures, development of a *C. difficile* management team and antimicrobial stewardship (Muto et al 2005). As a quality improvement initiative, checklists facilitated implementation and adherence of new policies and practices. Hospital rates of *C. difficile* decreased from 7.2 cases per / 1,000 discharges during the year before institution of these measures to 4.8 cases per / 1,000 discharges in the subsequent 5 years.

3 AIMS OF THE STUDY

The purpose of the present study was to investigate efficacy of different treatment modalities in recurrent *Clostridium difficile* infection and to characterize clinical feature and risk factors of extra-intestinal *Clostridium difficile* infection.

The specific aims were:

- I** Compare *Clostridium difficile* immune whey (CDIW) with metronidazole for treatment of recurrent CDI
- II** Investigate the efficacy of fecal transplantation in treatment of recurrent CDI
- III** Assess the effectiveness of rifaximin in recurrent CDI
- IV** Characterize clinical features and risk factors of extra-intestinal CDI

4 PATIENTS AND METHODS.

4.1 PATIENTS

In Study I eligible patients were at least 18 years of age and had experienced at least 2 episodes of mild to moderate CDI within 3 months. During the second or later episode the subjects were randomized to the study. All patients had to have symptoms of CDI and were required to demonstrate a positive *C. difficile* toxin EIA assay, which was confirmed in Helsinki University Central Hospital Laboratory Diagnostics, HUSLAB. Written informed consent was obtained from all patients. The patients inclusions and exclusion criteria are characterized in detail in the original publication. Patients were recruited from December 2004 to March 2006 at 10 centres in Finland. The centres were situated at Helsinki University Central Hospital (HUCH); Division of Infectious diseases, Central Hospital of Lapland, Rovaniemi, Turku University Hospital; Department of Internal Medicine, Helsinki City Hospital; Laakso Acute Care Hospital; Koskela Acute Care Hospital; Suursuo Hospital, Länsi-Uusimaa Hospital, Tammisaari, Oulu City Hospital, Turku Health Centre, and Satakunta Central Hospital, Pori.

In Study II all patients treated by FMT through colonoscopy were included in 5 hospitals: HUCH, Turku University Central Hospital, Satakunta Central Hospital, Turku Municipal Hospital, and Helsinki Municipal Hospital, from November 2007 through February 2010. The criterion for FMT was laboratory-confirmed RCDI (positive culture and toxin) despite antimicrobial treatment for CDI. All patients were refractive to standard therapy, and FMT was used as a salvage therapy after attempts of conventional therapy had failed. Only patients who had received FMT through colonoscopy according to the predetermined protocol using colonoscopy were included in the study. All the participating centres had electronic patient records including patient history, laboratory findings, and official information on the survival of the patient, which facilitated a reliable review.

In Study III all the patients who received rifaximin (Rifacol; Prodotti Formenti Srl, Milano, Italia) in HUCH, from March 2007 through December 2011 for CDI were included. Rifaximin was given only for RCDI. Information gathered from electronic patient records included patient history, laboratory findings, and official information on the survival.

In Study IV patients with extra-intestinal CDI were searched for in the electronic data files of the Division of Clinical Microbiology, HUSLAB. The laboratory which receives microbiologic samples from a population of 1.5 million analyzed all samples from patients that got the expenses covered by the community. The inclusion

criterion was detection of *C. difficile* in an extraintestinal sample analyzed between January 2002 and September 2012. Individual patient records were evaluated retrospectively in the archives of our hospital district.

4.2 STUDY DESIGNS

Study I was a controlled, double-blind, randomized, parallel-group, multicentre, comparative trial of CDIW and metronidazole in the treatment of RCDI. The duration of the active period was 14 days in all patients. Patients were randomized to receive either CDIW 200 ml t.i.d and metronidazole placebo tablets t.i.d, or CDIW placebo liquid 200 ml t.i.d. and metronidazole tablets 400 mg t.i.d. The study consisted of 4 visits and 2 follow-up contacts. All patients were seen by an investigator on d 0, 7, and 14 and were seen or contacted by telephone on day 28 and 70. *C. difficile* culture and toxin test was performed on d 0, 14, and 28. Patients kept a daily stool and symptom diary for 42 days.

Study II and III were a retrospective review of patients. The symptoms were evaluated 12 weeks after the start of treatment and patient records were followed up until one year after the treatment.

In Study IV extra-intestinal CDI were searched for in an electronic database of all *C. difficile* positive isolates found during a 10-year period. The medical records were reviewed retrospectively. Disease severity and co-morbidities of the patients were evaluated using Horn disease severity and Charlson co-morbidity indexes.

4.3 DEFINITIONS

In Study I the patient was considered to be clinically cured if he or she became asymptomatic during the treatment course. Clinical failure was defined as persistent or recurrent symptoms and signs, and a need for new therapy during treatment or follow-up period.

In Studies II and III a treatment failure was defined as persisting diarrhea with a positive *C difficile* toxin stool test.

In Study IV the severity of the disease was rated using the Horn index (Horn et al.1983). The Horn index is described in Table 3 (page 24). The severity of the underlying disease was scored using the Charlson index (Charlson et al. 1987), Table 5. Points were added from each of the below listed co-morbidities.

Table 5 Age-adjusted **Charlson** index.

One point	Myocardial infarction, congestive heart failure, peripheral vascular disease, dementia, COPD, connective tissue disease, peptic ulcer disease, uncomplicated diabetes mellitus, mild liver disease. Age 41 to 50 years.
Two points	Diabetes mellitus (if end-organ damage), moderate to severe chronic kidney disease, hemiplegia, leukemia, malignant lymphoma (solid tumor). Age 51 to 60 years.
Three points	Moderate to severe liver disease. Age 61 to 70 years.
Four points	Age 71 or older
Six points	AIDS

4.4 STUDY TREATMENTS

In Study I patients were treated either with CDIW or metronidazole. CDIW was manufactured by immunization of cows in their gestation period with inactivated *C. difficile* vaccine. The resulting colostrum was processed, immunoglobulins were concentrated and the end-product containing high titres of *C. difficile* immunoglobulin was used as CDIW. The production process is characterized in detail in the original publication. Whey made from colostrum from unimmunized cows was used as a negative control, which consistently gave no activity against *C. difficile*. Metronidazole and metronidazole placebo were both in the form of white capsules.

In Study II 70 patients were treated by FMT, which was performed via colonoscopy after colonic lavage. The patients were pretreated with vancomycin or metronidazole until a reduction of symptoms occurred. This treatment was discontinued an average of 36 hours before the transplantation. Colonic lavage was performed by oral ingestion of 4 L of a polyethylene glycol solution (Colonsteril; Orion Oyj, Espoo, Finland). During endoscopy no evident contraindications for fecal transplantation could be observed in any of the patients prepared for the transplantation. Biopsy specimens were taken when considered appropriate by the endoscopist. The patients were given instructions for home cleaning and disinfection to reduce the possibility of *C. difficile* re-infection at home.

All donor stools were freshly passed. Sixty-one of the stool donors were close relatives or other household members. In the remaining 9 cases, family members were not eligible or available as donors, and a healthy volunteer donated the stool. There were no food restrictions or recommendations for donors. Preparation of donor stool and the patient for the procedure is presented in Table 2 in the original publication. Individuals who had not received antimicrobial therapy for the past

6 months and who did not have any intestinal symptoms were considered to be suitable for stool donation. Preferred stool donors were as follows: (1) relatives, (2) individuals who had intimate physical contact with the patients (spouse or significant partner), or (3) any other healthy donors. Our protocol for donor screening is summarized in Table 1 in the original publication.

In Study III standard rifaximin treatment was oral rifaximin 400 mg twice a day for 14 days that was preceded by an oral course of vancomycin 125 mg four times daily for 14 days. This standard treatment was given to 25 patients. One patient got vancomycin tapering for 6 weeks before the oral course of rifaximin. Three patients got metronidazole 400 mg three times daily for 14 days instead of vancomycin before rifaximin. One patient got both metronidazole and vancomycin before the rifaximin therapy. Two patients got oral rifaximin 400 mg twice a day for 28 days without any immediately preceding courses of vancomycin or metronidazole.

4.5 MICROBIOLOGICAL METHODS

In Study I toxin test used in each centre was confirmed in the Helsinki University Laboratory, HUSLAB, by *C. difficile* toxin EIA assay (Premier Toxins A & B; Meridian Bioscience).

In Study II *C. difficile* culture and toxin test were done by each centres own laboratory. Strain typing of isolated *C. difficile* colonies was performed using the DiversiLab system (bioMérieux, Marcy l'Etoile, France). This method is based on PCR amplification of repetitive extragenic palindromic sequences and it reliably can distinguish *C. difficile* ribotype 027 strain from other strains.

In Study III *C. difficile* isolation was performed by culturing fresh stool samples on *C. difficile* selective CCFA agar (cycloserine-cefoxitin-fructose-egg yolk agar) at 35° C for 42 h in anaerobic atmosphere. Colonies with typical morphology, fluorescence, and odour were identified as *C. difficile*. Toxin production was analysed directly from faecal samples with the Premier Toxins A&B-test kit (Meridian; Bioscience Inc., Cincinnati, OH, USA) during 2007–2010 and with VIDAS *C. difficile* Toxin A & B CDAB-system (bioMérieux, Marcy l'Etoile, France) from 2011 onwards according to the manufacturer's instructions, or if the direct toxin test was negative, from *C. difficile* colonies. Strain typing was performed by DNA analysis using multiplex PCR. Antibiotic susceptibilities for metronidazole, vancomycin, and rifampin (reflecting the susceptibility for rifaximin) were determined with E-tests (bioMérieux) on Fastidious Anaerobe Agar (FAA)-plates (LabM, Lancashire, UK).

In Study IV *C. difficile* was isolated in extra-intestinal sites using conventional anaerobic bacteriologic techniques. These were not specifically designed to detect *C.*

difficile but rather designed to detect all anaerobic microbes. After transportation in Stuart transportation media the samples were streaked on FAA-plates (Lab M, Bury, United Kingdom) that were supplemented with 5% defibrinated horse blood. The plates were then incubated in anaerobic jars at 35°C. Isolation of *C. difficile* from peripheral blood samples was performed using the BacT/ALERT® Culture Media system for blood samples (bioMérieux, Marcy l'Etoile, France). Isolation of *C. difficile* in stool samples was performed using *C. difficile* selective CCFA at 35°C for 42 h in anaerobic atmosphere. Colonies with typical morphology, fluorescence, and odor were presumptively identified as *C. difficile*. Bacterial isolates were identified by biochemical tests. *C. difficile* toxins were detected directly from *C. difficile* colonies by the Premier Toxins A&B-test kit (Meridian; Bioscience Inc., Cincinnati, OH, USA) during 2007–2010 according to the manufacturer's instructions. From 2011 onwards the toxin genes were analyzed from *C. difficile* colonies with multiplex PCR (Antikainen et al. 2009). Strain typing when done was performed by DNA analysis using multiplex PCR (Antikainen et al. 2009).

4.6 ETHICAL ASPECTS

In Study I, the protocol and consent form, which was conducted in accordance with the declaration of Helsinki and all applicable laws and regulations, were approved by the Finnish Agency for Medicines (EudraCT 20004-000499-16) and by an institutional review board of the ethics committee at each of the centres.

In Study II, FMTs and the retrospective review of the patient records were approved by the institutional review boards of all the participating centres. All patients were informed about the experimental nature of this treatment procedure and about the available results in other previously published reports and possible risks of the procedure. All of the patients provided informed consent.

Studies III and IV were approved by the Institutional Review Board of HUCH.

5 RESULTS

5.1 PATIENT CHARACTERISTICS (I-III)

Patient characteristics and response to therapy are described in Table 6. In Study I total of 40 patients underwent randomization and 38 were included in the modified intention-to-treat population. Two patients were excluded from the efficacy analysis, one patient because the *C. difficile* toxin test was negative and another because the patient died on the second day on CDIW. However, the latter patient was included into the safety part of study and reported as a serious adverse event. All 38 patients were compliant with therapy, defined as >90% adherence to the assigned 14-d treatment regimen.

In Study II the mean time between the diagnosis of CDI and the initial stool transplantation was 133 days (range, 46–360 days). The patients had an average of 4.5 courses of antibiotics for CDI before FMT (range, 2–12). These treatments included a variety of metronidazole, vancomycin, and rifaximin regimens, and one patient also received intravenous immunoglobulin therapy. The baseline characteristics varied slightly according to the hospital. In one tertiary care university hospital there were more young patients (5 of 11 were younger than age 40), and all were outpatients. In secondary care municipal hospitals the patients were, on average, older, and there also were inpatients. Most patients had received antibiotics commonly associated with the risk of developing CDI, remarkably often cephalosporins (47 of 70). One nurse developed an occupational *C. difficile* strain 027 infection after having taken care of a patient with a 027 strain. There were 2 peripartum CDIs.

None of the 70 patients had definitive signs of IBD at colonoscopy. In some patients microscopic evaluation showed mild epithelial damage, edema, and scattered neutrophilic infiltrate. One patient was found to have adenocarcinoma of the colon at colonoscopy. Her *C. difficile* non-027 infection resolved after FMT but she died of the carcinoma 3.5 months after the transplantation.

In Study III the preceding infections and antibiotics before the initial CDI are given in Table 1 in the original publication. Antibiotic susceptibilities were determined of isolates from 22 patients. Most isolates (15 of 22, 68%) had very low MIC-values for rifampin (<0.002 lg/mL). Those strains with the DNA profile compatible with the virulent 027 ribotype had, on the average, more than 10-fold higher MIC for rifampin than other strains (Table 2 in the original publication). The average MIC of metronidazole was approximately 2-fold higher in 027 strains

than other strains. There was no evident difference in the average susceptibilities to vancomycin between 027 and non-027 strains.

Table 6 Patient characteristics and treatment outcomes of the Study (I-III) patients

	CDIW (Study I)	Metronidazole (Study I)	FMT (Study II)	Rifaximin (Study III)
No. of patients	18	20	70	32
Age mean	56	66	73	58
Age, range	22-85	25-92	22-90	19-88
Gender, male	7 (39%)	11 (55%)	28 (40%)	13 (41%)
Gender, female	11 (61%)	9 (45%)	42 (60%)	19 (59%)
Number of previous CDI episodes(range)	2.39 (1-4)	2.15 (1-4)	3.5 (1-12)	4.3 (2-12)
027 strain	NA*	NA*	36/70=51 %	8/27= 30%
Outpatient	17/18	18/20	60/70	NA
Response to therapy **	10/18 (56%)	18/20 (55%)	66/70 (94%)	17/32 (53%)

** In Study I two of the total 38 patients (5%) had a 027 strain (unpublished)

* in Study I 10 weeks, in Study II-III 12 weeks

5.2 RESPONSE TO THERAPY

Summary of the response to therapy is described in Table 6. In Study I the response rates by primary end-points to study drugs are shown in Table II in the original publication. No statistically significant differences were observed across the 2 treatment groups. None of the patients on metronidazole and 3 patients on CDIW received an open label treatment for CDI during the 2-weeks study medication period. After the treatment period and during the 70-d follow-up, 5 patients in the CDIW group and 9 in metronidazole group experienced a relapse, which needed an open-label re-treatment of CDI. At the end of the study 8 of 18 patients (44%) with CDIW and 9 of 20 (45%) with metronidazole experienced relapse. Kaplan-Meier estimates of relapse-free survival are shown in Figure 2 in original publication.

There were no differences between the study groups with regard to need of hospitalization. Of 13 patients hospitalized, CDIW was used in 7 and metronidazole in 6. The period of hospitalization was 4.8 and 4.5 days, respectively. Readmission

to hospital because of CDI was needed for 1 patient in the CDIW group and 2 in the metronidazole group. On day 14, eradication of *C. difficile* (toxin negative) was demonstrated in 6 of 15 patients (40%) receiving CDIW and in 11 of 18 patients (61%) receiving metronidazole. On day 20, six of 11 (55%) patients of the CDIW group and 10 of 13 (77%) of the metronidazole group showed eradication.

In Study II during the first 12 weeks of follow-up evaluation after the FMT resolution of symptoms was seen in all 34 (100%) patients with CDI due to a non-027 strain. Of the 36 patients with CDI due to a 027 strain, 32 (89%) had a favourable response. All 4 nonresponders with 027 *C. difficile* infection had serious conditions and died 1.5–3 months after the FMT. One patient had an especially severe CDI and was offered colectomy as a treatment option. He refused surgery and FMT was used as a salvage therapy. The second patient with a severe CDI had an incomplete pretransplantation lavage, did not have any response to FMT, and died of CDI 2 months after the FMT. The third patient had chronic obstructive pulmonary disease and developed severe diarrhea and died of CDI. The common feature of these 3 patients was an especially aggressive CDI due to ribotype 027. The fourth patient had end-stage myeloma. She had RCDI after antibiotic treatments for pneumococcal septicemia and meningitis shortly after FMT. She got the second FMT 24 days after the first procedure but subsequently died of myeloma related uremia. In study II during the 1-year follow-up period, 4 patients with an initial favourable response had a relapse after receiving antibiotics for unrelated causes. Two of these patients were treated successfully with another FMT and 2 were treated successfully with antibiotics for RCDI.

In Study III during 12 weeks follow up period, 17 (53%) patients had no relapse. There was a trend that those patients (75%, 6 of 8) who had *C. difficile* 027 strain tended to have a relapse more frequently than those who had a non-027 strain (42%, 8 of 19), but this trend was not statistically significant ($P = 0.11$, Fisher exact test). MIC value of rifampin predicted the response to rifaximin treatment ($P = 0.0461$, Mann–Whitney U-test). All the 12 patients who were cured had isolates with rifampin MIC values below 0.3 µg/mL, whereas 5 of the 10 patients who failed had a MIC value above this value ($P = 0.010$ Fisher exact test). When the epidemiological cut-off value of 0.004 µg/mL was used, which is considered to distinguish wild type *C. difficile* isolates from isolates with reduced susceptibility to rifampin according to EUCAST (The European Committee on Antimicrobial Susceptibility Testing -<http://www.eucast.org>), we get an OR 4.62 (95% CI: 0.52–65.4, $P = 0.172$) of risk of failure.

During the subsequent year, none of the 17 patients who responded to rifaximin developed CDI. Of the 15 who had a failure after rifaximin therapy, 8 patients recovered after receiving faecal transplantation. Two patients recovered after receiving a course of metronidazole. Four patients recovered after receiving one

or repeated courses of vancomycin. One patient ultimately recovered after receiving continuous vancomycin for 3 years.

5.3 ADVERSE EVENTS

In Study I adverse events were recorded in 10 of 20 patients receiving metronidazole and 12 of 18 patients receiving CDIW. The adverse events were mild and did not cause the cessation of study drugs. Only 2 adverse events were likely to be related to the investigational medicine, both in the metronidazole group, one metallic taste and one nausea. Two serious adverse events (SAE) were reported. Both were in the CDIW group. A patient with complicated diabetes and heavy coronary artery disease succumbed and 1 patient was readmitted to hospital because of *Escherichia coli* bacteraemia. None of these events was thought to be related to the study drug. In both cases, the causality of SAEs was classified by the reporter as unlikely to be due to the investigational product.

In Study II no immediately evident complications of FMT were observed. There were no reported transmitted infections. Four patients infected with the ribotype 027 strain did not respond to FMT and died within 3 months. No evidence could be shown that the death of these patients could have been caused or facilitated by intestinal lavage, colonoscopy, or FMT. Except for these 4 patients, none of our patients had any severe adverse events that could be related to FMT. In addition to these 4 patients, 10 patients died of unrelated illnesses during the 1-year follow-up period.

In Study III no obvious side effects were observed during the rifaximin therapy.

5.4 EXTRA-INTESTINAL CDI

We found altogether 31 patients (only 0.17% of all CDIs) with extraintestinal CDI in the databases during the 10-year study period. Examination of the patient records revealed that the patients could be categorized in to five different infection types: bacteremic infections (2 patients), abdominal infections without any prior surgery (4 patients), abdominal infections after surgery (7 patients), perianal abscesses (4 patients) and wound infections (13 patients). The number of extra-intestinal CDIs ranged from none to six cases per year. In most instances (26 out of 31, 85%) *C. difficile* was isolated together with other microbes. Strain typing was done of 9 isolates and 2 (22%) of these had a DNA profile compatible with the 027 ribotype.

The majority (24 out of 31, 77%) of patients had a Horn index 3 or 4 indicating a severe or fulminant disease. Many patients had severe underlying diseases and the mean Charlson index was 5.2. All patients had received antibiotics before developing an extra-intestinal CDI. Majority (81 %) of patients developed the infection when hospitalized. One-year mortality rate was 36% (11/31). One –year mortality was associated with both the Horn ($p=0.01$) and Charlson indexes ($p<0.001$). Patients with perianal abscesses tended to be younger and had lower Horn and Charlson indexes than the other patients ($p<0.05$, ANOVA). Nine patients (29%) were alcohol abusers to the extent that they had severe adverse effects related to alcohol abuse such as liver cirrhosis or pancreatitis. All the two patients with bacteremic infections had diarrhea and 14 of the remaining patients had clinical symptoms of a manifest diarrhea.

6 DISCUSSION

6.1 CDIW AND METRONIDAZOLE FOR RECURRENT CDI

CDIW is a nonantibiotic modality, which is based on orally ingested specific *C. difficile*-enriched bovine immunoglobulins. The present study was the first randomized, double-blind study where CDIW was compared to standard treatment. The results of this study demonstrate that the efficacy of CDIW in the treatment of mild-to moderate recurrent CDI seems to be similar to that of metronidazole (sustained recovery 56% vs. 55%).

The effect of bovine antibody-enriched whey on CDI has been previously investigated (van Dissel et al. 2005, Numan et al. 2007) in Netherlands. In an open uncontrolled study, 101 patients (109 CDI episodes) received after completion of at least 10 days of standard antibiotic treatment (metronidazole or vancomycin), daily treatment with immune milk for 2 weeks (Numan et al. 2007). The follow-up period lasted 60 days. 64% of patients suffered their first episode of CDIW. In total, 11 (10%) of 109 episodes were followed by a relapse of CDI. In contrast to that study, our study was a double-blind and controlled study, using a colostrum-based product instead of immune milk, and CDIW in the absence of antibiotics. Compared to the latter study (Numan et al. 2007) the present study was also more homogeneous with regard to patient material, all patients suffering from recurrent CDI. Thus the 2 studies are not comparable in terms of patient selection, the planning of the studies, use of previous antibiotic treatment, and immune whey product.

45% of patients of either the CDIW or the metronidazole group failed. This degree of failure may reflect the feature of the study population that many of the patients included into our study had more than 1 recurrence of CDI. Nevertheless, the results were in line with a previous report of antibiotic treatment of recurrent CDI (McFarland et al. 2002).

The mechanism of action of CDIW is unclear. The specific antibodies may bind toxins or prevent mucosal damage caused by toxins. CDIW may prevent the adhesion of *C. difficile* to mucous membranes. However, the mode of action of metronidazole and CDIW are different. CDIW as an immunomodulatory does not directly inhibit the growth of *C. difficile*. CDIW is an interesting alternative to conventional antibiotic treatment in patients with recurrent CDI. CDIW does not alter normal colonic bacterial flora as antibiotics do and does not cause resistance problems.

6.2 FECAL MICROBIOTA TRANSPLANTATION FOR RECURRENT CDI

FMT is a strategy that restores the diversity of the gut microflora, which may confer protection against toxigenic *C. difficile*. In Study II 70 patients with RCDI were treated with FMT and followed up retrospectively for one year. Sixty-six of 70 patients (94%) recovered, which is an outstanding result in a patient group refractory to other treatment methods. In Study I patients, who had, on average, a milder disease of RCDI, only 55% recovered with metronidazole and 56% with CDIW during a 70-day follow-up period. Study II also included patients who were treated successfully with FMT after having failed rifaximin or intravenous immunoglobulin therapy.

C. difficile ribotype 027 is associated with a more severe diarrhea and with more recurrences (Goorhuis et al. 2008). In a recent study, a new macrocyclic antibiotic, fidaxomicin, was compared with vancomycin in the treatment of CDI (Louie et al. 2011). CDI recurred significantly less often with fidaxomicin than with vancomycin (15% vs. 25%) during 4 weeks of follow-up evaluation. However, among patients with *C. difficile* ribotype 027 infection, fidaxomicin appeared to be no better than vancomycin in preventing recurrences. The recurrence rates were 24% and 23%, respectively. In the present study, only 4 (11%) of our 36 patients with *C. difficile* ribotype 027 infection developed a recurrence during the 12 weeks of follow-up evaluation after FMT as compared with the recurrence rate of 24% after the fidaxomicin treatment after 4 weeks of follow-up evaluation in the previous study. Although the characteristics of the patients may be different in our study as compared with that of the fidaxomicin study and, therefore, the results are not directly comparable, our study shows that FMT is an effective treatment option for recurrent CDI and also for recurrent CDI caused by the virulent *C. difficile* ribotype 027.

Four of our patients had CDI diarrhea despite FMT and died shortly after the transplantation. All 4 of these patients were seriously ill already before FMT and the procedure was used as salvage therapy. Up to a 23% overall mortality rate has been reported with CDI at 30 days (Pepin et al. 2005³). Thus, the mortality rate in our study does not appear to be greater than in some previously reported series, suggesting that FMT itself seems to be a rather safe procedure. FMT seems to be safe also for patients with underlying serious conditions. Our study included one immunosuppressed lung transplantation patient who had a favourable response after FMT. Also, a patient with a fulminate life-threatening CDI has been treated successfully with FMT (You et al. 2008).

Performing FMT by colonoscopy has the advantage that it enables differential diagnostics of long lasting diarrhea, for example, to exclude IBD, which is a risk

factor for CDI (Mylonaki et al. 2004, Issa et al. 2008, Rodeman et al. 2007) and to detect diverticulosis of the colon and colon carcinoma, which may be masked by CDI. The lavage before the colonoscopy conceivably results in a reduced colonic biomass, which may have facilitated the restoration of the colonic bacterial flora by the transplant. We recommend using fresh instead of frozen donor stool transplant because bacteria are presumably more viable in fresh stool, despite good results that also have been published using a frozen donor stool transplant (Jorup-Ronstrom et al. 2006). There have been concerns regarding the risks, including perforation induced by colonoscopy in patients with active colitis. All of our patients were pretreated with antibiotics for CDI, which probably reduced inflammation, and possibly also the risk of perforation at the time of colonoscopy. The colonoscopy technique used seemed to be safe because none of our patients had any severe immediate adverse events that could be related directly to colonoscopy.

The principal potential risk associated with FMT is transmission of contagious agents contained in the donor stool. There are risks of transmitting agents that do not cause a disease immediately after transplantation, but may complicate the treatment of the patient in the future. Such agents may include multidrug-resistant gram-negative bacteria. We suggest that tests be conducted to detect possible multidrug-resistant bacteria in donor stool to maximize the safety of the procedure. Despite evident risks associated with FMT, no reported transmitted infections or significant immediate adverse effects have been reported to date. Although we did not systemically search for transmitted infections in our patients, we have so far not found any clinical evidence of transmitted infections related to the fecal transplant.

6.3 RIFAXIMIN FOR RECURRENT CDI

The outcome after rifaximin in patients with RCDI was promising as 17 (53%) of the 32 patients responded favourably. Although the treatment in almost all patients was preceded by a course of vancomycin or metronidazole, rifaximin most likely contributed to the cure, as there was an association between the susceptibility of *C. difficile* isolates to rifampin and clinical outcome. Nevertheless, it should be noted that in our series, the course of metronidazole or vancomycin, that immediately preceded the administration of rifaximin, probably had an important role in the cure rate. Rifaximin therapy seemed to be safe as none of our patients experienced any notable side effects during rifaximin treatment.

Despite this, our results were not as effective as reported previously for rifaximin in CDI. In an earlier prospective trial on patients with mild-to-moderate CDI, all 16 patients (100%) who completed treatment with rifaximin recovered from the infection (Basu et al. 2010). In another series of 8 patients with RCDI who received

rifaximin, 7 patients (88%) experienced no further diarrhoea recurrences and the patient who had a recurrence subsequently responded favourably to a second course of rifaximin treatment.(Johnson et al. 2007). In yet another study of 6 patients with recurrent CDI who received rifaximin, 4 (66%) patients had no further diarrhoea episodes (Johnson et al. 2009). Finally, in a randomized placebo-controlled trial where rifaximin was given immediately after standard antimicrobial therapy for CDI, 26 (79%) of the 33 patients responded favourably (Garey et al. 2011). Taken together, the overall cure rate of 85% of all the 63 patients in the above four studies is clearly higher than the cure rate of 53% of our 32 patients.

It is possible that our patients were on the average more prone to relapses, which might have contributed to a less favourable response as compared with the results reported previously. All our patients who received rifaximin had RCDI. This was because our standard treatment for the first episode of CDI is metronidazole or vancomycin, which is consistent with current recommendations (Bauer et al. 2009, Cohen et al. 2010). There may have been a selection in our series for those patients who were more prone to have relapses. Increased age, initial disease severity, and hospital exposure have been predicted to predict CDI recurrence (Gujja and Friedenber g 2009, Eyre et al. 2012), the use of proton pump inhibitors have been implicated as a risk factor for the development of CDI (Bavishi and DuPont 2011) and factors such as increased age, low serum albumin level and high serum creatinine level have been associated with the severity of the disease (Khanna et al. 2012¹, Ananthakrishnan et al. 2012). We attempted to analyse whether such factors would reveal any association with the success of rifaximin treatment, but our series was too small to address this.

Of note was that the MIC value of rifampin, although all the MIC values of our strains were rather low, seemed to predict the response to rifaximin treatment. This may imply that sensitivity testing may be used to select patients with extremely low MIC values for rifaximin treatments. On the other hand, there was a correlation between the 027 strain type of *C. difficile* and an increased MIC value of rifampin as well as to metronidazole that have also been observed in earlier studies (Hecht et al. 2007, Citron et al. 2009, Huang et al. 2010, Goldstein et al. 2011). Thus, specific pathogenic factors of the 027 isolates may be responsible for the difference in the cure rate and not necessarily the low MIC value. Nevertheless, rifaximin may be more efficacious against 027 strains with extremely low MIC values of rifampin than against other strains.

Development of resistance against rifaximin is a potential concern. Prior exposure to rifamycins has been reported to be a risk factor for rifampin-resistant CDI (Curry et al. 2011) and rifaximin resistant *C. difficile* strains may emerge even during therapy (Johnson et al. 2009, Carman et al. 2012). Although the rate of rifaximin resistance among *C. difficile* isolates has been as low as 2% (Jiang 2010),

the rate of resistance may reach 20% (Miller et al. 2011²) and rifamycin-resistant *C. difficile* strains have been reported to cause hospital outbreaks (Curry et al. 2009). Of concern are also reports that suggest that the antibiotic resistance of *C. difficile* may be changing to the worse (Spigaglia et al. 2011). These issues on resistance restrict the use of rifaximin in CDI and highlight the importance of sensitivity testing of *C. difficile* isolates. Despite this, rifaximin has a reasonable effect in CDI and it can be considered as an optional treatment for RCDI.

6.4 EXTRA-INTESTINAL CDI

Extra-intestinal CDIs were rare. Only 31 cases were found during the 10-year period studied in our hospital district that provides health care to a population of ca. 1.5 million. The number of cases was low (0.17% of all CDIs) as compared to all patients with CDI during the same time period. The numbers of extra-intestinal *C. difficile* cases observed would translate to a mean yearly incidence of 0.2 per 100.000. This may, however, be an underestimate as *C. difficile* may be difficult to isolate and the rate of recovery may vary depending on the isolation method (Carrol and Bartlett 2011). It should be noted that *C. difficile* was isolated in most cases together with other microbes, which may make the isolation of *C. difficile* even more demanding. Detection of *C. difficile* in extra-intestinal sites remains a challenge and emphasizes the need for sensitive microbiologic detection methods.

A common feature of extra-intestinal CDI was that most patients were hospitalized and had received antibiotics, which increase the risk of *C. difficile* carriage. The patients may or may not have clinical symptoms of diarrhea but many had severe comorbidities, previous surgery, or intestinal infection. Such characteristics have also been observed in previous reports (Wolf et al. 1998, Gerard et al. 1989). Most of the patients in our series had high Charlson co-morbidity and Horn disease severity indexes, which reflect the morbidity of our patients. Mortality was high and it correlated with these indexes.

A significant proportion of our patients were alcohol abusers. Alcohol abuse has been reported also in previous reports on extra-intestinal CDIs (Garcia-Lechuz et al. 2001, McGill et al. 2011). It has recently been found that a subgroup of alcoholics has dysbiosis with an altered colonic microbiota with lower median abundances of *Bacteroidetes* and higher ones of *Proteobacteria* (Mutlu et al. 2012). Lower abundances of *Bacteroidetes* have also been observed in the intestinal flora of patients with CDI enteritis (Manges et al 2010) suggesting some similarity in the intestinal flora of alcohol abusers and patients with CDI. Such changes in the intestinal microbiota of alcohol abusers may increase the risk of *C. difficile* colonization. It is also possible that alcohol abuse or diseases due to alcohol abuse

may suppress immune responses, which could increase the risk of *C. difficile* colonization and infection.

Treatment of CDI enteritis is shifting towards local therapy such as to the use of per oral non-absorbable antibiotics vancomycin, rifaximin, and fidaxomicin as well as to the use of FMT (Lo Vecchio and Zacur 2012, Pepin et al. 2007) . The occurrence of deep extra-intestinal CDIs implies that there is still need for systemic antibiotics in selected cases of severe *C. difficile* colitis.

It can be concluded that extra-intestinal CDIs are rare and occur in hospitalized patients who often have severe co-morbidities. *C. difficile* is usually isolated together with other microbes but may also be the single microbe of the infection. Most extra-intestinal CDIs are localized in the abdominal area and result either from intestinal perforation after infection or leakage after surgery. *C. difficile* wound infections may result from contamination by feces. *C. difficile* can cause bacteremia and it may enter distant sites through transient bacteremia.

6.5 REMAINING CHALLENGES

Treatment of CDI has relied primarily on metronidazole and vancomycin for the past 30 years. Limitations of these agents have stimulated the development of newer therapies. In the present study we have investigated the treatment of recurrent CDI with antibiotics (metronidazole and rifaximin), passive immunotherapy (CDIW) and bacteriotherapy with FMT. Although these treatment modalities were not directly compared to each others, FMT appears to be the most potent treatment of recurrent CDI. Sixty-six of our 70 patients (94%) recovered with FMT. Our results are similar to previous data on FMT with mean cure rates approximately 91 % (Gough et al. 2012).

In spite of growing interest in FMT, many questions and challenges remain. Comparative studies are needed to address which route of transplantation is the most appropriate for various presentations of CDI. In addition, future metagenomic studies of colonic microbiota may reveal clues of the specific microbes or microbe classes required to suppress CDI. There are risks of transmitting agents that do not cause a disease immediately after transplantation, but may complicate the treatment of the patient in the future. Long-term studies after FMT need to be performed to address transmission of contagious agents contained in the donor stool and other possible long term side-effects.

Recent advances suggest that effective donor microbiota can be produced by repeated filtering, washing, freezing and even freeze-drying feces – and yet still deliver a clinical result equivalent to that of fresh crude fecal homogenate when treating CDI (Hamilton et al. 2012, Hamilton et al. 2013) while removing the fecal odour.

The use of feces may be eliminated in favour of defined mixtures of cultured bacteria that confer colonization resistance against *C. difficile*. In the future, lyophilized full spectrum human donor microbiota or mixtures of key bacteria may be available in capsule formulation to treat CDI and perhaps be used routinely after antibiotic usage to prevent gut flora damage.

Active rather than passive immunization is an attractive goal for effective and durable protection against CDI. Both passive immunity with monoclonal antibodies and vaccines are currently undergoing clinical trials involving humans. Preliminary evidence shows higher serum antitoxin antibody levels in vaccinated patients than in those exposed naturally to CDI (Aboudola et al 2003). Vaccine was used in combination with antibiotics to successfully treat three patients with RCDI (Sougioultzis et al 2005). Major questions regarding the immune response to vaccine in elderly populations, the magnitude and duration of vaccine protection, and the selection of an appropriate at-risk population for vaccination remain to be answered.

7 SUMMARY AND CONCLUSIONS

The main results of the present study can be summarized:

- I** Efficacy of CDIW in the treatment of mild-to moderate RCDI seems to be similar to that of metronidazole. CDIW did not cause systemic or local side-effects.
- II** FMT through colonoscopy seems to be an effective treatment for RCDI and also for RCDI caused by the virulent *C. difficile* 027 strain. Colonoscopy is a feasible technique for FMT, which seems to be safe also for patients with underlying serious conditions.
- III** Rifaximin has a reasonable effect in CDI and it can be considered as an optional treatment for RCDI. Rifaximin may be more efficacious against non-027 strains with extremely low MIC values of rifampin than against other strains.
- IV** Extra-intestinal CDIs occur mainly in hospitalized patients with significant comorbidities. Most extraintestinal CDIs are localized in the abdominal area and result either from intestinal perforation after infection or leakage after surgery. *C. difficile* can cause bacteremia and may enter distant sites through transient bacteremia. *C. difficile* wound infections may result from contamination by feces. Mortality of extra-intestinal CDIs is high and associated with underlying diseases .

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